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TOXICITY OF ALUMINIUM IN SEAWATER: DIATOM SENSITIVITY

Megan Gillmore

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The overall aim of this study was to relate aluminium sensitivity for three species of marine diatoms (*Ceratoneis closterium, Minutocellus polymorphus,* and *Phaeodactylum tricornutum*) to aluminium speciation and investigate the mechanisms of toxicity of aluminium to the diatoms. The three diatoms differed in physical characteristics to investigate whether differences in sensitivity were influenced by biotic factors, such as the ultrastructure of the cell wall, cell shape or cell size. In addition total and dissolved (0.45 µm and 0.025 µm filtered) aluminium in the bioassays were measured over time to determine the forms of aluminium associated with observed effects.

There was a varied response in growth rate sensitivity to aluminium between diatom species with the most sensitive species being *C. closterium* (72-h exposure inducing 10% inhibition of growth rate (72-h IC10) = 80 (55-100) µg Al/L (95% confidence limits)) followed by *M. polymorphus* (72-h IC10 = 540 (460-600) µg Al/L) and *P. tricornutum* (72-h IC10 = 2100 (2000-2200) µg Al/L) based on time averaged concentrations of measured total aluminium. Toxicity in the most sensitive diatom, *C. closterium*, was shown to be due to the dissolved aluminium forms of aluminate ($Al(OH)_4^-$) and aluminium hydroxide ($Al(OH)_3^0$), while both dissolved and precipitated aluminium contributed to toxicity in *M. polymorphus*. In contrast, aluminium toxicity to *P. tricornutum* was due to precipitated aluminium. Interspecies differences in aluminium sensitivity were not related to cell size or cell shape. A relationship between the ultrastructure of cell walls and aluminium toxicity was observed, with the two diatoms with a siliceous cell wall being more sensitive than the diatom with the weakly siliceous cell wall. Sensitivity of *C. closterium* and *M. polymorphus* was influenced by initial cell density, with aluminium toxicity increasing with increasing initial cell density. No effects on plasma membrane permeability were observed for any of the species suggesting mechanisms of aluminium toxicity to diatoms do not involve compromising the plasma membrane.

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TOXICITY OF ALUMINIUM IN SEAWATER: DIATOM SENSITIVITY

By

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Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the degree of Honours Bachelor of Environmental Science (Advanced). It does not include any material published by another person without due reference within the text. The field and laboratory work presented in this thesis was performed by the author, except where acknowledged. This thesis has not been submitted for a degree at any other university.

Megan Gillmore

28th October 2014

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Abstract

Although recognised as a nonessential metal, aluminium was long considered virtually innocuous to marine organisms, largely because of the very low concentrations of aluminium naturally occurring in the marine environment. It is now clear however, that human activities are contributing to elevated concentrations of aluminium in industrialized harbours and coastal waters of Australia, which have the potential for adverse impacts on marine organisms. Toxicity testing recently conducted on a suite of marine algae, invertebrates and fish to derive a new ANZECC water quality guideline trigger value for the protection of organisms from aluminium identified marine diatoms (Bacillariophyceae) were amongst the most sensitive species to aluminum.

The overall aim of this study was to relate aluminium sensitivity for three species of marine diatoms (*Ceratoneis closterium, Minutocellus polymorphus,* and *Phaeodactylum tricornutum*) to aluminium speciation and investigate the mechanisms of toxicity of aluminium to the diatoms. The three diatoms differed in physical characteristics to investigate whether differences in sensitivity were influenced by biotic factors, such as the ultrastructure of the cell wall, cell shape or cell size. In addition total and dissolved (0.45 µm and 0.025 µm filtered) aluminium in the bioassays were measured over time to determine the forms of aluminium associated with observed effects.

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1 INTRODUCTION

1.1 Aluminium occurrence in the marine environment

1.1.1 Natural sources and concentrations

Aluminium is the most abundant metal and the third most abundant element in the earth's crust, constituting approximately 8.1% by weight (Wilson, 2011). Despite this abundance, dissolved aluminium is typically found at trace concentrations in the marine environment, typically less than 1 µg Al/L (Table 1.1). Aluminium is not present as a pure metal in the environment, but instead occurs in combination with silicon and oxygen as complex aluminosilicate and oxide minerals (Namieśnik and Rabajczyk, 2010). The relatively small portion of dissolved aluminium in natural waters compared to its overall abundance in the terrestrial environment can be in part explained by the low solubility of these minerals in the pH range characteristic of most natural waters (pH 6-8) (Figure 1.1). Consequently very little aluminium dissolves as a result of weathering under normal conditions (Gensemer and Playle, 1999; Namieśnik and Rabajczyk, 2010). Release of dissolved aluminium can be substantially enhanced however, when environments are subjected to periodic or sustained exposure to strong acidifying inputs (Gensemer and Playle, 1999).

Table 1.1 Typical concentrations of aluminium found in ocean surface waters

Location	Concentration range (µg/L)	Reference
North Atlantic Ocean - North Sea,	0.5-5.5	Hydes and Liss (1977)
North Atlantic Ocean - Canary Basin	0.32-0.67[a]	Kramer et al. (2004)
Mediterranean Sea	0.5 – 1.5	Caschetto and Wollast (1979)
Pacific Ocean	0.027 – 0.22 [a]	Measures et al. (2005)
Southern Ocean - Weddell Sea	0.027-0.13 [a]	Moran et al. (1992)
Southern Ocean	0.019 [a]	Middag et al. (2011)
Arctic Ocean	0.081-0.11 [a]	Moore (1981)

[a] Original data presented in nM

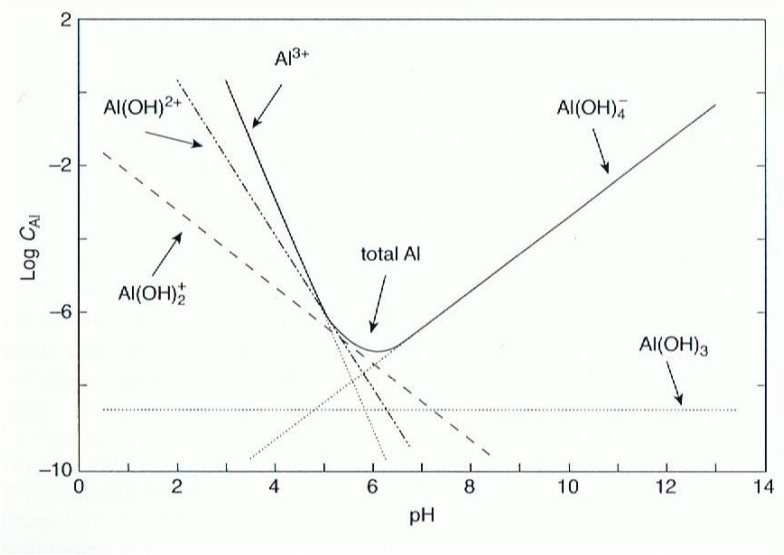


Figure 1.1 Solubility of individual aluminium species and total aluminium in water as a function of pH. Reprinted from vanLoon and Duffy (2011), temperature not provided

The major factor mediating the concentration and distribution of aluminium in the marine environment is the balance between its external sources, removal processes and internal cycling. The high concentration of dissolved aluminium typically found in river waters (1-27 µg/L, Brown et al. (2010)) prompted early researchers to propose fluvial inputs as the dominant source of dissolved aluminium to the marine environment (Stoffyn and Mackenzie, 1982), however, fluvial inputs are now recognised to be negligible due to estuarine removal processes such as flocculation and sedimentation (Hydes and Liss, 1977; Mackin and Aller, 1984; Maring and Duce, 1987). Hydes and Liss (1977) showed that scavenging of dissolved aluminium occurs in estuarine waters via aluminium-organic complexation and the flocculation of colloidal clay minerals. Moreover, Mackin and Aller (1984) showed that undisturbed estuarine sediments will act as a sink for dissolved aluminium because of diffusion across the sediment-water interface and adsorption of aluminium within the sediment.

The vertical distribution of aluminium in the marine environment provides a good indication of its dominant external sources and removal mechanisms within the marine environment. The vertical profile distribution of dissolved aluminium typically exhibits a surface concentration maximum, a mid-depth minimum and concentrations increasing towards the sediment-water interface (Orians and Bruland, 1986; Bruland and Lohan, 2003). Dissolved aluminium concentrations of the North Pacific and the North Atlantic exhibit this vertical profile (Figure 1.2). An exception to the typical profile is observed in the Mediterranean Sea where surface waters typically do not demonstrate the surface maximum (Figure 1.2); the reason for this has not yet been elucidated, and has been described as "mysterious" (Pilson, 2013). It is widely accepted that the predominant source of dissolved

aluminium to the ocean is from the partial dissolution of wind-blown dust from arid and semi-arid regions of the continents, as evidenced by the surface concentration maximum and higher concentrations observed in the surface waters of the North Atlantic, a region that receives substantial input form the Saharan dust plume, relative to the North Pacific (Orians and Bruland, 1986; Maring and Duce, 1987; Measures et al., 2005). The small increase in dissolved aluminium in deep waters with close proximity to the sediment-water interface supports a further minor input of dissolved aluminium to the ocean from the desorption or dissolution of sedimentary particles and flux to the overlying waters (Orians and Bruland, 1986).

In addition, the typical vertical profile of dissolved aluminum supports a scavenged type distribution; that is, strong interactions with particles and a short oceanic residence time (Bruland and Lohan, 2003). Several studies have demonstrated that removal of aluminium in the ocean occurs via active biological uptake by diatoms (Stoffyn, 1979; Moran and Moore, 1988a; Moran and Moore, 1988b; Gehlen et al., 2002) and passive scavenging onto either living or non-living particulate matter (Hydes, 1983; Orians and Bruland, 1986). Gehlen et al. (2002) provided evidence for aluminium being removed from seawater through its incorporation into diatoms during silica frustule biosynthesis. The residence time of aluminium varies from 4 weeks to 4 years in surface waters, and from 50 to 150 years in deep waters where scavenging rates are considerably reduced (Orians and Bruland, 1986). Although aluminium is abundant in the terrestrial environment, the insolubility of the parent material and the balance between external inputs and removal processes are responsible for its relatively low natural occurrence in the marine environment.

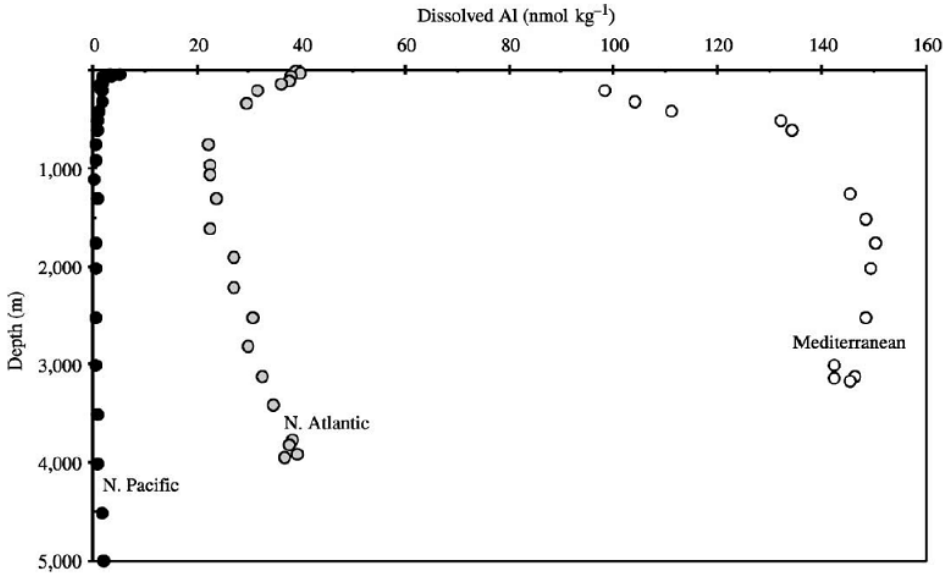


Figure 1.2 Vertical profiles of dissolved aluminium for the North Pacific (Orians and Bruland, 1986), North Atlantic (Hydes, 1979) and the Mediterranean Sea (Hydes et al., 1988). Reprinted from Bruland and Lohan (2003)

1.1.2 Anthropogenic sources and concentrations

While the evidence supports that natural sources of aluminium to the marine environment are the dominant transport route, anthropogenic inputs are a potential localized source of aluminium to the marine environment and impacted coastal waters. Apte et al. (1998) has shown that the concentration of trace metals along NSW coastal waters did not vary substantially from open ocean concentrations in the absence of anthropogenic inputs, however, this may not be the case for coastlines that receive major river inputs. Dissolved aluminium concentrations ranged from 1.3 to 7.3 µg/L in coastal waters of NSW (King, 2013), while industrialized harbours and coastal waters of Port Curtis, QLD, Australia reached concentrations of 21-83 µg/L (Angel et al., 2012) (Table 1.2). The major anthropogenic sources of concern include discharges associated with alumina and aluminium production, the dredging-induced resuspension of sediments containing aluminosilicates and the disturbance or drainage of acid sulfate soils for coastal development particularly along the north east Australian coast (Wilson and Hyne, 1997; Angel et al., 2012). Acid sulfate soils release aluminium into coastal waters through the process of oxidation of sulfidic material exposed to the atmosphere resulting in acidification, followed by leaching of sediment-bound metals such as aluminium (Nordmyr et al., 2008). While an anthropogenic concern, this process is also known to occur naturally during exposure of acid sulfate soils to the atmosphere during ebb tides.

1.1.3 Chemical speciation in marine waters

One of the major challenges of aluminium research is that aluminium occurs in a variety of chemical species which are present as dissolved, amorphous and solid phases and undergo dynamic transformations from one phase to another. The concentrations of these phases are of prime importance when assessing the toxicological impacts. Aluminium is an amphoteric element and thus the dominant phase changes markedly with pH (Figure 1.3). In the marine environment (pH 8.0-8.3) aluminium speciation is dominated by the aluminate anion ($Al(OH)_4^-$), with minor contributions from the soluble neutral aluminium hydroxide complex ($Al(OH)_3^0$) (Millero et al., 2009; Brown et al., 2010). An important distinction between aluminium speciation in marine waters compared to freshwaters is the absence of cationic species (Al^{3+}, $AlOH^{2+}$ and $Al(OH)_2^+$) which dominate under acidic pH conditions (Wilson, 2011).

Table 1.2 Dissolved aluminium concentrations measured around Sydney NSW (King, 2013) and Port Curtis QLD (Angel et al., 2012) Australia

Year	Dissolved aluminium concentration (µg/L)
Sydney, NSW	
Milsons Point Wharf	3.7
Neutral Bay Wharf	3.9
Cremorne Point Wharf	3.3
Taronga Zoo Wharf	3.2
Clifton Gardens	3.0
Kyeemagh Beach	2.7
Silver Beach, Kurnell	2.7
Foreshore Rd Jetty, Botany Bay	7.3
Oak Park	1.3
Port Curtis, QLD	
2012	< 1 -21
2004	< 0.6 - 44
2003	< 0.6 - 83

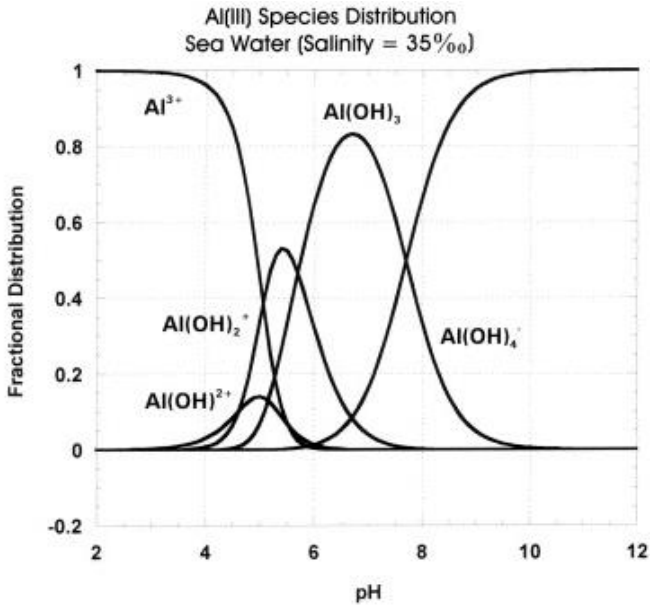


Figure 1.3 Speciation modelling for aluminium in seawater over a range of pH values from 2 to 12. Reprinted from Elkins and Nelson (2002)

Moreover, a recent study by Angel et al. (2014) has shown that aluminum speciation in seawater is driven by its solubility limit and solubility kinetics. Their study found that at pH 8.15 and 21°C, aluminium speciation was dynamic in the short-term, but appeared to approach equilibrium after 28 days, with a solubility of approximately 500 µg/L. Above this concentration, aluminium began to precipitate and these precipitates dominated speciation at high total aluminium concentrations. In addition, above the solubility limit, dissolved aluminium concentrations increased above the solubility limit in the short term and then decreased over longer time scales, with larger decreases for higher total aluminium concentrations (Figure 1.4). These changes in dissolved aluminium indicate that kinetic and thermodynamic factors prevent the establishment of equilibria between dissolved and precipitated aluminium forms in the short term. Practically the dynamic nature of dissolved aluminum introduces many problems for standard toxicity tests (typically 2-5 days), particularly in understanding the concentration that organisms are exposed to (Golding et al., 2014). Furthermore, an important implication is that marine organisms may be exposed to either the anionic and neutral species of dissolved aluminium at concentrations below 500 µg/L, or a combination of both dissolved and precipitated species above this (Angel et al., 2014). Therefore, knowledge of the form of aluminum is required if we are to understand the extent to which each form contributes to the toxicity in marine organisms.

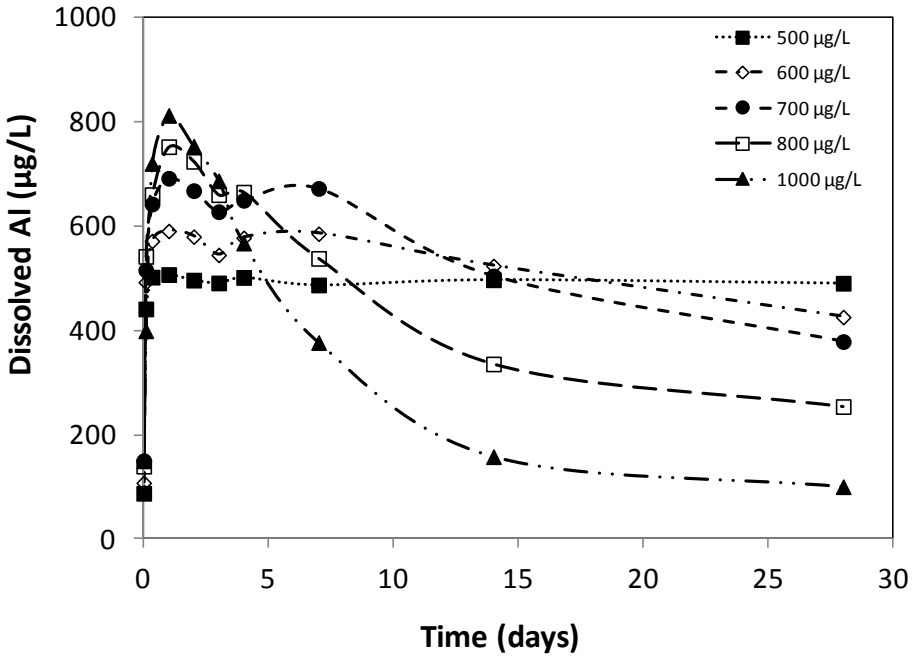


Figure 1.4 The concentration of dissolved aluminium (<0.45 µm) as a function of time in seawater for total aluminium of 500 to 1000 µg/L. Reprinted from Angel et al., (2014)

There are currently no analytical methods to fractionate the dissolved forms ($AlOH_4^-$ and $Al(OH)_3^0$) in seawater. Therefore, aluminium speciation is operationally defined by size fractionation of dissolved and precipitated forms. Filtration is most commonly used to operationally define the dissolved portion of trace metals. For the purposes of this thesis, dissolved (<0.025 µm) aluminum was defined as that which passes through a 0.025 µm filter and includes truly soluble species and low molecular weight forms of aluminium. Dissolved (<0.045 µm) aluminium was defined as that which passes through a 0.45 µm filter and includes colloidal (defined here as small particles that pass through a 0.45 µm filter but not a 0.025 µm) as well as the low molecular weight forms of aluminium. The term total aluminium refers to aluminium in all forms (from truly soluble species through to precipitated forms).

1.2 Toxicity of aluminium to marine organisms

Although recognised as a non-essential metal, aluminium was long considered virtually innocuous to marine organisms, in large part because of the very low concentrations of aluminum naturally occurring in the marine environment. It is now clear however, that human activities are contributing to elevated concentrations of aluminium occurring in industrialized harbours and coastal waters of Australia, which have the potential for adverse impacts on marine organisms. Concern from both industry and regulatory agencies has recently prompted toxicity testing on a suite of marine algae, invertebrates and fish to derive a new Australian and New Zealand Environment and Conservation Council (ANZECC) water quality guideline value for the protection of organisms from aluminium in marine waters (Golding et al., 2014). Aluminium exposure concentrations which caused chronic toxicity in marine organisms and were used in the calculation of the revised Australian and New Zealand guidelines are presented in Table 1.3.

Acute toxicity tests generally have short durations relative to the test organism's life span and typically investigate lethal endpoints (ANZECC/ARMCANZ, 2000). In contrast, chronic toxicity tests run for longer duration, typically over a number of generations or at least a significant portion of the organisms' life span, and measure sub-lethal endpoints such as growth rate, reproductive output and changes in feeding behavior and respiration (ANZECC/ARMCANZ, 2000). Chronic toxicity data are preferred over acute toxicity data for guideline development, as they are more appropriate to achieve the overall aim of the Guidelines to provide life-long protection for aquatic organisms and hence, it is assumed, for aquatic ecosystems (Warne et al., 2014). Traditionally, no observable effect concentrations (NOECs) have been used for the derivation of water quality guidelines. The NOEC is defined as the highest test concentration where there is no statistically significant effect relative to

the control. There is a recognised need to move away from the use of hypothesis test based values, such as NOECs as they are totally dependent on the concentrations used in tests, and to more reliable low effect measures of toxicity such as the concentrations that cause 10% effect on a population (e.g. 10% inhibition in population growth, IC10) (Warne et al., 2014). To derive a water quality guideline of sufficient reliability chronic toxicity data for at least 8 species that belong to 4 taxonomic groups is recommended (Warne et al., 2014). The toxicity data presented in Table 1.3 was used to derive the aluminium water quality guideline, of 24 µg/L providing protection for 95% of marine species. These bioassays were conducted in natural seawater, at a pH characteristic of natural conditions and used measured total aluminium concentrations in the calculation of toxicity endpoints. Older literature values (not shown in Table 1.3) used to derive the original ANZECC/ARMCANZ (2000) environmental concern level of 0.5 µg/L were based on nominal (designated) aluminium concentrations rather than measured concentrations in the calculation of acute rather than chronic toxicity endpoints and did not consider the speciation of aluminium in marine waters. Such data are ranked low in qualifying for use in deriving a "high reliability" guideline and further justified the need for robust toxicity data in the recently derived water quality guideline (Golding et al., 2014).

There is great interspecies variability in aluminium toxicity to marine organisms (Table 1.3). It is difficult to generalize which taxonomic groups are most tolerant or sensitive to aluminium exposure from the small number of species studied so far, but limited evidence has provided the following trends. Among the marine invertebrates that have been investigated the sea urchin, *Heliocidaris tuberculata* (no effect on embryo development up to 28000 µg Al/L), and the corals, *Acropora tenuis* (IC10 (fertilisation) at 2793 µg Al/L), exhibited high tolerance for aluminium. Studies also indicate that there are several marine algal species with high aluminium tolerances, including the brown algae, *Hormosira banksia* and *Ecklonia radiate,* (72-h IC10 at 9800 and 6800 µg/L, respectively) and the green algae, *Tetraselmis* sp and *Dunaliella tertiolecta* (72-h IC10 at 3200 and 1400 µg/L, respectively). In contrast, the mollusc species and microalgae from the class Bacillariophyceae (diatoms) were amongst the most sensitive marine organisms to aluminium. The marine diatom, *Ceratoneis closterium* (72-h IC10 at 16 µg/L) was the most sensitive species to aluminium of all algae and invertebrate species investigated and therefore heavily influenced the guideline value.

Table 1.3 Chronic toxicity data from 6 taxonomic groups used to derive the high reliability guideline value for total aluminium in marine waters. Adapted from Golding et al. (2014). All experiments were carried out using natural seawater and at pH 8.2

Species	Duration and endpoint	Toxicity measure	Toxicity value (µg/L)
Microalgae - diatom			
Ceratoneis closterium[a]	72-h growth rate inhibition	IC10[c]	16 (3.2-78)
Minutocellus polymorphus	72-h growth rate inhibition	IC10	690 (580-800)
Mollusc - oyster			
Saccostrea glomerata[b]	48-h embryo development	NOEC[d]	100
Saccostrea echinata	48-h embryo development	EC10	410 (340-480
Mollusc - mussel			
Mytilus edulis plannulatus	72-h embryo development	EC10[e]	250 (240-260)
Cnidarian - coral			
Acropora tenuis	18-h larval metamorphsis	IC10	1300 (860-1700)
Microalgae – green			
Dunaliella tertiolecta	72-h growth rate inhibition	IC10	1400 (820-1900)
Tetraselmis sp	72-h growth rate inhibition	IC10	3200 (1100-5400)
Macroalgae – brown alga			
Hormosira banksii	72-h germination success	NOEC	9800
Ecklonia radiata	72-h germination success	IC10	6800 (5400-8200)
Echinoderm – sea urchin			
Heliocidaris tuberculata	72-h embryo development	NOEC	28000

Values in parentheses are 95% confidence limits

[a] *Ceratoneis closterium* was formerly known as *Nitzschia closterium*

[b] *Saccostrea glomerata* was formerly known as *Saccostrea commercialis*

[c] IC10/IC25/IC50 = aluminium concentration to cause 10, 25 and 50% inhibition in the population relative to control, respectively

[d] NOEC = No observable effect concentration

[e] EC10/EC50 = aluminium concentration to cause 10 and 50% effect in the population relative to control, respectively

1.3 Importance of marine microalgae in ecotoxicology

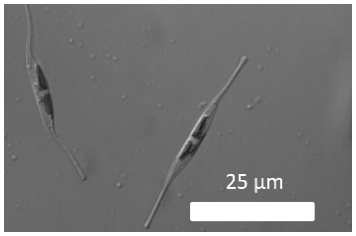
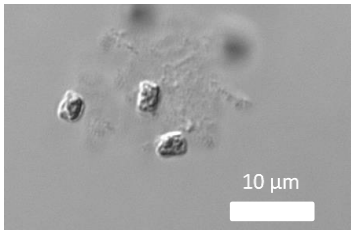
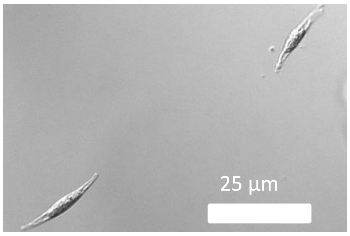
Microalgae occupy a pivotal position in the aquatic food web and the biogeochemistry of the oceans. As photosynthetic organisms they fix a major portion of the earth's carbon, generate oxygen and supply high-quality food for aquatic organisms (Dixit et al., 1992). Thus any adverse impact on the community structure of microalgae is likely to affect organisms at higher trophic levels and have important consequences for the health of the whole ecosystem (Stauber, 1995). For this reason studies focusing on the response of microalgae to contaminants have particular environmental relevance for hazard assessment and the development of environmental regulations (Stauber, 1995; Levy et al., 2007; Rodgher et al., 2012).

Microalgae make ideal test species because they can be easily cultured in the laboratory, providing abundant population numbers and genetically uniform cultures for toxicity testing (Stauber, 1995). Their response to toxicants are also highly reproducible (Stauber, 1995). Microalgae are ideally suited to flow cytometric analysis as they are single-celled and contain photosynthetic pigments such as chlorophyll *a*, which autofluoresce red when excited by blue (488 nm λ) light (Stauber et al., 2002). Flow cytometric analysis can determine growth-rate inhibition, changes in cell size and effects on chlorophyll *a* fluorescence (Adams and Stauber, 2004). Growth-rate inhibition toxicity tests are the most widely used of all algal tests as they have the advantage of detecting more subtle effects on population, rather than mortality (Stauber, 1995; Franklin et al., 2001).

Microalgal growth-rate inhibition tests are useful for establishing cause-effect relationships for particular toxicants, but are limited in that they do not provide any indication of the mechanism of toxicity (Stauber et al., 2002). To overcome this limitation, flow cytometric analysis can be coupled with the use of specific fluorescent dyes to detect metabolic changes in cells (e.g. enzyme inhibition or plasma membrane permeability) and provide further information about the mechanisms of action of toxicants (Stauber et al., 2002; Adams and Stauber, 2004). For example, plasma membrane integrity can be evaluated by detecting enhanced fluorescence in cells stained with the fluorescent dye SYTOX Green. SYTOX Green is a nucleic acid stain that is cell impermeable, but will passively diffuse into cells with a permeabilized plasma membrane (Sato et al., 2004; Tashyreva et al., 2013). When SYTOX Green binds to DNA in cells with a compromised plasma membrane it fluoresces a green colour (emission maximum of 523 nm) due to the dye uptake when excited with a 450-490 nm λ laser (Sato et al., 2004; Tashyreva et al., 2013). Its bright fluorescence in the green spectral region allows its observation simultaneously with the red auto-fluorescence of photosynthetic pigments such as chlorophyll *a* (Tashyreva et al., 2013). The major advantage of this is that membrane permeability and growth-rate inhibition can be evaluated simultaneously.

Diatoms, in particular, are a useful and convenient class of microalgae for toxicity testing as they are abundant world-wide and present in all aquatic environments (oceans, lakes, rivers, wetlands etc.) with sufficient light (Dixit et al., 1992). At present the cause of interspecies differences in toxicity to metals, are not well understood (Levy et al., 2007; Levy et al., 2008; Debelius et al., 2009). To increase our knowledge of the sensitivity of diatoms, which have been identified as a particularly vulnerable class to aluminium exposure, three marine diatoms differing in ultrastructure, cell shape and cell size have been selected for further investigation (Table 1.4).

Table 1.4 Marine diatom species investigated in the current study: culture medium used, cell features and representative image

Diatom species	***Ceratoneis closterium* (formerly *Nitzschia closterium*) (Ehrenb.) W. Smith**	***Minutocellus polymorphus* (Hargraves and Guillard) Hasle, Von Stoch and Syvertsen**	***Phaeodactylum tricornutum* Bohlin**
Culture medium	Natural seawater f medium	Natural seawater f/2 medium	Natural seawater f/2 medium
Cell shape	Pennate diatom	Centric diatom	Pennate diatom
Cell ultrastructure	Siliceous cell wall	Siliceous cell wall	Weakly siliceous cell wall
Place isolated	Port Hacking, NSW, Australia	Port Hacking, NSW, Australia	Unknown
Representative image			

1.4 Mechanisms of aluminium toxicity in marine microalgae

Few studies have examined the mechanism of aluminium toxicity to marine organisms and at present we have limited mechanistic understanding of why some marine species are sensitive to aluminium and others are tolerant. Research into the mechanisms of toxicity is especially important for the marine diatom *C. closterium* as this species has been identified as the most sensitive to aluminium in marine waters. Information on the mechanisms of toxicity of aluminium to freshwater microalgae is

more readily available but has focused on situations of low pH where aluminium speciation is dominated by cationic species (Al^{3+}, $AlOH^{2+}$ and $Al(OH)_2^{+}$). The interaction of cationic species of aluminium with the surface of aquatic organisms and their transport mechanisms through biological membranes (Monteiro et al., 2012) are not likely to be the same as those of the anionic or neutral aluminium species that are present at the pH and ionic composition of seawater. However, there are basic principles of direct and indirect mechanisms of toxicity that are common to all toxicants and can be used to speculate about mechanisms of toxicity relevant to aluminium speciation in seawater.

Interactions between metal ions and microalgae are known to occur at the cell plasma membrane surface (Campbell et al., 2002). Dissolved aluminum in marine waters may be directly toxic to microalgae by adsorption to compatible functional groups on the plasma membrane followed by interference of membrane function by compromising the integrity and porosity of the membrane. Alternatively aluminium transport across the plasma membrane may interfere with intracellular metabolic processes such as the disruption of enzyme functions via blocking specific functional groups and competing with essential metabolites for binding sites (Monteiro et al., 2012). It has been suggested that aluminate could be transported into the cell via anion membrane channels while the mechanism by which the neutral but polar dissolved aluminium hydroxide form may interact with the cell membrane is unknown (Golding et al., 2014). It has also been suggested that aluminium uptake may delay frustule dissolution rates in diatoms and thereby cell division (Gensemer and Playle, 1999). Smothering of the algal cells by excess aluminium hydroxide precipitate may reduce the transport of nutrients and gases to the algal cell surface and act as a form of photosynthetic shade thereby affecting growth rate. Aggregates of $Al(OH)_3$ precipitates around the algae may also act as a point source of highly concentrated dissolved aluminium during the initial phase of aluminium dissolution and adsorption above the limit of solubility. Indirect mechanisms of aluminium toxicity in marine waters may involve competition for binding sites with essential anionic nutrients such as phosphate, nitrate and silicate in the surrounding media.

When exposed to a toxicant or stressor microalgae may also employ a range of detoxification mechanisms such as binding or sequestration of metal ions at non-metabolically active sites or release organic exudates (i.e. biopolymers excreted from organism including glycolic acid, polysaccharides and carbohydrates) capable of complexation with metal ions that may extracellularly mask a toxic metal (Franklin et al., 2002; Levy et al., 2008; Monteiro et al., 2012). Other biotic factors such as initial cell density have also been found to influence metal toxicity. Franklin et al. (2002) found that copper toxicity decreased at higher initial cell densities due primarily to greater copper adsorption by algal cells resulting in depletion of the equilibrium concentration of dissolved copper in solution.

1.5 Project aims and objectives

The overall aim of this study was to relate aluminium sensitivity for three species of marine diatoms to aluminium speciation and investigate the mechanisms of toxicity of aluminium to the diatoms.

The specific objectives of this project were to:

i. Measure total and dissolved aluminium over the course of diatom bioassays to determine changes in aluminium speciation over the 72-h exposure period and how this relates to biological effects on diatoms
ii. Determine the effect of aluminium on diatom population growth using 72-h exposure bioassays
iii. Determine the influence initial cell density has on the sensitivity of marine diatoms to aluminium toxicity to assist in investigating mechanisms of toxicity
iv. Identify the optimal SYTOX Green staining conditions for investigating membrane permeability of the three diatoms present in this study and validate the method through confocal microscopy
v. Determine the effect of aluminium on permeability of the diatom plasma membrane using 72-h exposure bioassays and the fluorescent probe SYTOX Green.

2 MATERIALS AND METHODS

2.1 General laboratory methods

Due to the ubiquitous nature of aluminium in the environment, trace metal cleaning procedures were employed to avoid contamination. Plastic containers used for storing seawater, standards, stock solutions and samples for metal determinations were constructed of either high-density polyethylene, polycarbonate or polypropylene plastic. All plastic-ware was acid-washed before use to prevent any potential contamination occurring from surface desorption or leaching of aluminium from the plastic itself. Acid-washing procedures were equipment specific (summarised in Table 2.1) and carried out in a clean room environment.

Toxicity tests were performed in 250 mL borosilicate glass Erlenmeyer flasks, coated with a silanising solution (Coatasil, Ajax Finechem, Auburn, NSW, Australia) to reduce adsorption of aluminium to the glass. Flasks were acid-washed by soaking in 10% (v/v) HNO_3 (AR Grade, Merck, Darmstadt, Germany) for a minimum of 24 h, then rinsed 10 times with Milli-Q® water (18.2 MΩ/cm, Milli-Q®, Millipore).

All reagents were prepared using analytical grade chemicals and made up in high purity Milli-Q® water (Millipore). A copper stock of 5 mg L^{-1} was prepared volumetrically using copper (II) sulfate, ($CuSO_4.5H_20$, AR Grade, Ajax Finechem, Australia) and used to prepare copper reference treatments in each toxicity tests. The nutrients, nitrate and phosphate, were also prepared volumetrically at concentrations of 2.1 g L^{-1} $NaNO_3$ (AR Grade, Merck, Australia) and 0.22 g L^{-1} KH_2PO_4 (AR Grade, Ajax Finechem, Australia). Sodium hydroxide solutions were prepared gravimetrically at concentrations of 1.08, 0.108 and 0.02 M NaOH (AR Grade, Ajax Finechem, Australia).

Measurements of pH were performed using a Thermo Orion pH meter with an epoxy body Iodine/Iodide probe (meter model 420, probe model ROSS 815600, Thermo Fisher Scientific, USA) which was calibrated daily against pH 4.00, 7.00 and 10.00 buffers (Orion Pacific, Sydney, NSW, Australia).

Table 2.1 Cleaning procedures for plastic-ware used in experimental work

Equipment description	Equipment use	Acid-washing procedure
High density polyethylene containers (Nalgene)	Seawater collection and storage	Bottles were filled with 10% (v/v) nitric acid (HNO_3) (Tracepur, Merck) capped and allowed to stand for ≥ 24 h, then rinsed 10 times with Milli-Q® water (Millipore)
0.45 µm filter cartridge (Sartorius)	Seawater filtering	Through silicon tubing and filter cartridge 1 L of 10% (v/v) HNO_3 (AR Grade, Merck) was passed, followed by 4 L of Milli-Q® water (Millipore)
5 mL polypropylene vials, 30, 120 and 500 mL polycarbonate vials (Techno Plas)	Storage of samples for metal determinations; preparation and storage of stock solutions, standards and aluminium test solutions	Vials and lids were soaked in 10% (v/v) HNO_3 (Tracepur, Merck) for ≥ 24 h, then rinsed 10 times with Milli-Q water® (Millipore), and allowed to dry in a ISO-5 laminar flow hood
10 mL syringes (Terumo)	Sample delivery through 0.45 µm filters	Syringe piston was removed from cylinder and both pieces were soaked in 2% (v/v) HNO_3 (Tracepur, Merck) for ≥ 24 h then rinsed 10 times with Milli-Q® water (Millipore)
0.45 µm cellulose nitrate filter (Sartorius)	Subsampling dissolved aluminium	Using acid-washed 10 mL syringe, 10 mL of 4% HNO_3 (v/v) (Tracepur, Merck) was draw up and dispensed through filter followed by 3 dispenses of Milli-Q® water (Millipore) through filter
0.025 µm cellulose nitrate membrane filter (Millipore)	Subsampling dissolved aluminium	Filter paper placed in 10% (v/v) container of HNO_3 for ≥ 5 minutes, removed with teasers and transferred to Milli-Q® (Millipore) wash solution 1, then Milli-Q® wash solution 2
Polycarbonate filter unit (Sartorius)	For use with membrane filters	Polycarbonate filter unit was taken apart with plastic parts soaked in 10% (v/v) HNO_3 (Tracepur, Merck) and o-rings soaked in 2% (v/v) HNO_3 (Tracepur, Merck) for ≥ 24 h, then rinsed 10 times with Milli-Q water® (Millipore)
Pipette tips (0.2 mL, 1.0 mL and 5.0 mL)	Reagent and standard preparations; scratching and subsampling from bioassay flasks	Pipette tips were soaked in 10% (v/v) HNO_3 (Tracepur, Merck) for ≥ 24 h, rinsed 10 times with Milli-Q water® (Millipore), then allowed to dry in a ISO-5 laminar flow hood

2.2 Diatom cultures and growth conditions

Marine microalgae from the class Bacillariophyceae were originally obtained from the CSIRO Collection of Living Microalgae (Marine and Atmospheric Research, Hobart, Australia) and maintained at CSIRO, Centre for Environmental Contaminant Research (Sydney, Australia). *Ceratoneis closterium* (formerly *Nitzschia closterium* (Ehrenb.) W. Smith (strain CS-5, temperate)) was cultured in natural seawater f medium (Guillard and Ryther, 1962). *Minutocellus polymorphus* (Hargraves and Guillard) Hasle, Von Stosch and Syvertsen (strain CS-3) and *Phaeodactylum tricornutum* Bohlin (strain CS-29/4) were cultured in f/2 growth medium (half strength f medium, Guillard and Ryther (1962)). All cultures were maintained in a temperature-controlled room at 21°C on a 12:12 h light:dark cycle (Sylvania F20W/154-RS daylight fluorescent tubes; 70 µmol photons/m^2/s). All cultures were renewed weekly by inoculating 1.0 mL into freshly autoclaved media under sterile conditions. Cultures of *P. tricornutum* were axenic while *C. closterium* and *M. polymorphus* were not.

2.3 Flow cytometry

Flow cytometry analysis was performed using a Beckman Coulter, Cytomics FC500, Flow cytometer operated with CXP Software. Illumination was provided by an air-cooled Argon ion laser providing 20 mW at an excitation wavelength of 488 nm (blue light). Flow cytometry measures the physical properties of individual cells as they move in a fluid stream past a beam of light (Franklin et al., 2004). The light interacts with the cell and a group of sensors measure the light scattered by the cell and the fluorescence emission of the cell simultaneously. Shifts in forward-angle light scatter (forward-scatter, < 19°) provides information about the particle's size while shifts in side-angle light scatter (side-scatter, 15-85°) provides information about the granular content within a particle (Stauber et al., 2002). Fluorescence was collected at a range of wavelengths by 5 photomultiplier tubes (FL1-FL5). Chlorophyll *a* autofluorescence was detected as red fluorescence in the 660 to 690 nm band (FL4) and SYTOX emission was detected as green fluorescence in the 515 to 535 band (FL1). The forward scatter signal was used as the acquisition threshold to exclude non-algal particles and dead algal cells. All parameters were collected as logarithmic signals and analyses were performed at a high flow rate (60 µL min^{-1}), with an acquisition of 120 s and >1000 bead events. The instrument was calibrated regularly using Flow-Check Fluorospheres (Beckman Coulter) to verify instrument performance. Final instrument settings for the FC500 are given in Table 2.2.

Table 2.2 Instrument settings for Beckman Coulter FC500 flow cytometer

Parameter	Setting		
Threshold			
Primary threshold	Forward scatter	Value: 10	
Secondary threshold	None		
Compensation	None		
Detector/Amps	*Voltage*	*AmpGain*	*Mode*
FS	889	2.0	Log
SS	0	1.0	Log
FL1	396	1.0	Log
FL2	252	1.0	Log
FL3	250	1.0	Log
FL4	350	1.0	Log
FL5	430	1.0	Log

Data were displayed and analysed using CXP Software and a specially built protocol to allow simultaneous determinations of cell density and membrane permeability. Determinations of cell density required the use of an internal standard (fluorescent beads, Flow-Count Fluorospheres provided in an aqueous medium, Beckman Coulter). Immediately before each cell density measurement, the fluorescent beads were vortex mixed and 25 µL was added to 500 µL of sample. The fluorescent beads were identified on a dot-plot of side-scatter vs FL2 and a region of interest created around the population. This population was then gated out (procedure for eliminating unwanted particles) from a dot plot of side-scatter vs FL4 to obtain algal cells alone. A region of interest was created around the population of healthy control cells on the side-scatter vs FL4 plot. The analysis region was wide enough to include shifts in these parameters as a result of toxicant exposure. Region statistics were used from both plots to determine the number of beads and algae events in each regions. From this, absolute cell concentrations (cells/mL) were determined using the following equation:

$$\frac{No.of\ events\ in\ region\ containing\ cell\ population}{No.of\ events\ in\ bead\ population} \times \frac{No.of\ beads\ (in\ 25\mu L\ of\ bead\ stock)}{Volume\ of\ sample\ (mL)}$$

$$= Concentration\ of\ algal\ population\ (cells/mL)$$

Determinations of membrane permeability utilised the nucleic acid stain SYTOX Green. The staining conditions appropriate for assessing membrane permeability of the three marine diatoms required optimisation (section 2.5.1). The FL1 channel was used to measure cellular green fluorescence of the SYTOX Green stain. A two dimensional dot plot of FL4 vs FL1 was created and gated to only include the cell population region displayed in the dot-plot of side-scatter vs FL4. A one-dimensional histogram was also created to plot the cell number vs the FL1 intensity also gated to only display the cell population region in the dot plot of side scatter vs FL4. Qaudrants (E1, E2, E3 and E4) were set on the dot-plot and markers (M1 and M2) were set on the histogram to identify any increase or decrease in FL1 fluorescence intensity, relative to the control. An increase in green fluorescence intensity in FL1 from E1 to E2 or from M1 to M2 was indicative of cells with a permeabilized plasma membrane. Based on the summary statistics produced for each plot type, the number of cells in the SYTOX Green regions (E2 or M2) as a percentage of the total number of cells was used to quantify the proportion of cells with permeable membranes.

2.4 Method development: diatom membrane permeability

2.4.1 Optimisation of diatom cell staining with SYTOX Green

To study the optimal SYTOX Green staining conditions (concentration and incubation time) algal cells in the exponential phase of growth (5 days) were harvested by centrifugation (700 g, 7 min, Spintron GT-175BR) and rinsed three times with 0.45 µm filtered seawater to remove nutrient-rich culture medium. Following the wash, the concentrated cell suspension was counted using flow cytometry and suspended at 1-3x10^5 cells/mL in filtered seawater (100mL) enriched with nutrients (15 mg/L NO_3^- as $NaNO_3$ and 1.5 mg/L PO_4^{3-} as KH_2PO_4).

The parent stock solution of SYTOX Green (5 mM in dimethylsulfoxide (DMSO), Invitrogen, USA) was stored at -20°C in the dark to prevent photo-degradation. A 500 µM intermediary stock solution of SYTOX Green was prepared by adding 10 µL of the parent stock to 90 µL of Milli-Q water (Millipore) in an Eppendorf tube. This intermediary stock solution was then further diluted with Milli-Q water to produce working stocks of 1-40 µM SYTOX Green. Working stocks were prepared gravimetrically in acid-washed 5 mL polypropylene vials (Table 2.3). All SYTOX Green stock solutions were kept at -4°C in the dark.

Table 2.3 Preparation of SYTOX Green working stock concentrations (1-40 µM) from 500 µM intermediary stock.

Desired Concentration (µM)	Volume of 500 µM stock to add (µL)	Volume of Mill-Q water (µL)	Final Volume (µL)
1	10	4990	5000
10	20	980	1000
20	20	480	500
30	30	470	500
40	40	460	500

A positive control was prepare by transferring an aliquot of the algal cell suspension to a glass tube and heat treating (60°C, 5 min) in a water bath to cause the plasma membrane to become permeabilized. The heat-treated suspension was cooled to room temperature and combined 1:1 with the untreated cell suspension (negative control) to produce a suspension containing both cells with permeabilized plasma membranes and cells with intact plasma membranes. Mixtures of heat-treated and non heat-treated cells were utilised in the optimisation of the SYTOX green method.

The optimal concentration of SYTOX Green was determined by adding SYTOX Green (25 µL) to 500 µL of mixed cell permeability suspensions in duplicate with a 5 min incubation in the dark. Final concentrations of SYTOX Green were 0.05, 0.5, 1, 1.5 and 2 µM. To find the optimal SYTOX Green incubation time, the mixed cell permeability suspension was stained with SYTOX Green (0.5 µM final concentration) in duplicate and incubated for 5, 10, 15, 30 and 60 min in the dark. Following incubation, samples were analysed on the flow cytometer using the FL1 channel to measure cellular green fluorescence of the SYTOX Green stain.

The lowest dye concentration and shortest incubation time where all cells exhibited bright and even fluorescence with clear separation between the positive and negative control cell populations were considered the optimal staining conditions.

2.4.2 Validation of flow cytometry results with confocal microscopy

Cells were examined using confocal laser-scanning microscopy to confirm the SYTOX Green dye was only being taken up by cells with permeabilized plasma membranes. Positive and negative algal cell suspensions were incubated with 0.5 µM SYTOX Green for 5 min in the dark, centrifuged

(700g's for 4 min), and observed using a Leica TCS SP5 laser-scanning confocal microscope and a 63x oil immersion objective lens. The microscope is equipped with argon, krypton and helium/neon lasers and has 5 channels for simultaneous detection of five different fluorescent signals. SYTOX Green was excited with the 488 nm argon laser at 20% of the maximum laser intensity. The SYTOX Green emission was collected between 500 and 590 nm; chlorophyll fluorescence was collected between 615 and 700 nm.

2.5 Diatom bioassays

2.5.1 Preparation of aluminium test solutions

Aluminium test solutions were prepared according to the protocol developed by Angel et al. (2014). Aluminium chloride added to seawater undergoes hydrolysis resulting in a decrease in pH (Martin, 1991). Aluminium stock solutions were prepared in sodium hydroxide to minimise the pH of toxicity test bioassays decreasing upon spiking. Stock solutions were prepared in acid-washed 30 mL polycarbonate vials. Aluminium stock solutions of 1 and 10 g Al/L were prepared by adding 0.179 g and 0.895 g of $AlCl_3.6H_2O$ (AR Grade, Ajax Finechem, Australia) to 20 mL of 0.108 M NaOH and 10 mL of 1.08 M NaOH, respectively (Angel et al., 2014). A third solution of 0.01 g Al/L was prepared by diluting 0.1 mL of the 1 g Al/L stock to a final volume of 10 mL with 0.02 M NaOH. To limit changes in chemical speciation aluminium stock solutions were prepared on the day of toxicity test initiation.

Aluminium test solutions (10 to 10,000 µg Al/L nominal) were prepared in acid-washed 500 mL polycarbonate containers by adding appropriate aliquots (<1% of final volume to maintain pH and salinity) of the aluminium stock solutions to the filtered seawater. The nutrients, nitrate and phosphate (section 2.1), were added to give final concentrations of 15 mg NO_3^-/L and 1.5 mg PO_4^{3-}/L. The pH of the test solutions was measured and fine adjustments, with either 0.108 M or 1.08 M NaOH, were made to obtain a pH of 8.2 ± 0.05.

2.5.2 Toxicity test procedure

The chronic toxicity of aluminium to the three marine diatoms was determined using 72-h growth-rate inhibition bioassays. Bioassay methods were based on OECD Test Guideline 201 (2011) and the protocol of Stauber et al. (2005) and are summarised in Table 2.4. For each bioassay, 5 different aluminium treatments (10 to 10000 µg Al/L) and a control (aluminium-free filtered seawater plus nitrate and phosphate) were prepared. Copper was used as a reference toxicant to assess the performance and response of the diatom cultures over time to ensure that the algae were responding

to a known reference toxicant in a reproducible way (see Appendix A.1 for more detail). Triplicate 100 mL samples of the test solutions were dispensed into test flasks for each treatment and control. The test volume of 100 mL per flask was used to accommodate the volume of solution required for metal speciation analysis. Initial experiments were conducted to confirm that the change in volume from that in the standard protocol (50 mL) to 100 mL, did not alter diatom control growth rates or sensitivity to aluminium (see Appendix A.2 for more detail).

Table 2.4 Summary of 72-h growth-rate inhibition bioassay test conditions and protocol

Bioassay conditions	
Temperature	21 ± 2°C
pH	8.2 ± 0.5
Light quality	Cool white fluorescent lighting
Light intensity	100-120 µmol m^{-2} s^{-1}
Light cycle	12-h light: 12-h dark
Bioassay protocol	
Test type	Static
Test duration	72 h
Test chamber	250 mL glass Erlenmeyer flasks, silanised with Coastasil solution
Test volume	100 mL
Inoculum stock culture	6 days old
Initial cell density	2-4 x 10^3 cells/mL
Replication	3 replicates per treatment; ≥ 3 tests per diatom species
Test medium	0.45 µm filtered seawater + 15 mg NO_3^-/L and 1.5 mg PO_4^{3-}/L
Test endpoints	Growth-rate inhibition; membrane permeability
Test acceptability	≥ 1 doublings/day growth rate in controls and < 1 unit change in pH units in control treatments

Algal cells in the exponential phase of growth (5-6 days) were harvested from cultures for use in bioassays following a repeated process of washing three times with 0.45 µm filtered seawater and centrifugation (4 x 700 g, 7 min, Spintron GT-175BR) to remove culture medium and exudates. Following the wash, the concentrated cell suspension was counted using flow cytometry and an inoculum prepared to obtain a final cell density of 2-4 x 10^3 cells/mL in the bioassay. This relatively low initial cell density was used to better simulate algal cell densities found in surface seawaters and avoid changes in chemical speciation, partitioning and subsequent bioavailability, which may occur when high cell densities are used (Franklin et al., 2002; Stauber et al., 2002).

Flasks were covered with lids and incubated for 72 h on a 12:12 h light/dark cycle (Phillips TLD 30W/33; 100-120 µmol photons /m^2/s) at 21°C. Test flasks were rotated and shaken twice daily by hand to ensure sufficient gas exchange. The pH was recorded initially and after 72 h. Sub-samples of the test solution were collected from each replicate flask daily for metal analyses. Algal cell densities and membrane permeability were measured concurrently every 24 h using flow cytometry.

The test was acceptable if the control growth rate was ≥ 1 doubling/day, the control growth rate coefficient of variation was < 10%, and if pH changes were < 1 unit over the 72-h incubation period (Stauber et al., 2005). Bioassays for *M. polymorphus* and *P. tricornutum* were repeated twice, while *C. closterium* was repeated three times.

2.5.3 Influence of initial cell density

To determine the influence of initial cell density on the toxicity of aluminium to the three diatoms, 72-h bioassays were conducted at 2 or 3 different initial cell densities at the approximate IC25 or IC50 concentration of aluminium (Table 2.5). Algal cell harvesting, as well as temperature and light conditions were identical to those used in the diatom bioassays. Test flasks were rotated and shaken twice daily by hand to ensure sufficient gas exchange. The pH was recorded initially and after 72 h. Algal cell densities were determined daily by flow cytometry. Test solutions were sub-sampled (5 mL) from each flask daily for total (unfiltered) and dissolved (<0.45 µm) aluminium analysis, and dissolved (<0.025 µm) at 72 h to determine if the form of aluminium differed between the different cell density treatments. For example, changes in aluminium complexation or colloid size distribution may occur if higher cell densities result in increased cellular exudates being dispensed into solution. Sub-samples were preserved with 5% and 2% HNO_3 (v/v) (Tracepur, Merck) for total and dissolved aluminium respectively.

Table 2.5 Initial cell densities investigated and measured total aluminium concentration tested

Diatom species	Initial cell densities	Total Al concentration
Ceratonesis closterium	3.1×10^3 ; 2.8×10^4	850 µg/L (IC50)
Minutocellus polymorphus	3.9×10^3; 3.9×10^4 ; 1.3×10^5	1750 µg/L (IC50)
Phaeodactylum tricornutum	4.3×10^3; 4.0×10^4 ; 1.4×10^5	3890 µg/L (IC25)

2.5.4 Statistical analysis of toxicity data

Bioassays for each diatom species were conducted ≥ 3 and ≥ 2 times for effects on growth rate inhibition and cell membrane permeability, respectively. The growth rate (cell division rate) was the slope derived from a linear regression of $\log_{10}$ cell density versus time (h). Growth rates for treatment flasks were expressed as a percentage of the control growth rate.

The 72-h IC10 and IC50 values (i.e. the aluminium concentration to reduce the growth rate by 10% and 50%, respectively, after 72 h compared to the controls) was calculated using linear interpolation of pooled data from individual bioassays (ToxCalc Version 5.0.23C, Tidepool Scientific Software, McKinleyville, CA, USA). The data were tested for normal distribution using Kolmogorov D Test ($p > 0.01$) and equal variances using Bartlett's test ($p = 0.08$). Measured dissolved (<0.45 µm) and total aluminium concentrations at the time of test initiation were used in calculations of toxicity endpoints. Toxicity endpoints were also calculated using measured time-averaged dissolved (<0.45 µm) and total aluminium concentrations for comparison. Time-averaged aluminium concentrations were calculated using a Microsoft Excel program and averaging aluminium concentrations measured at 24-h intervals over the bioassay (0, 24, 48 and 72 h), assuming linear change between each time point.

For the initial cell density experiments, algal growth in the aluminium treatment was expressed as a percentage of the control. After the data had been tested for equal variances (Bartlett's test, $p = 0.08$), a Tukey-Kramer multiple-comparison test was used to determine which initial cell densities were significantly ($p < 0.05$) different.

2.6 Aluminium analyses

2.6.1 Chemical subsampling

Test solutions were sub-sampled (5 mL) from each flask daily for total (unfiltered) and dissolved (<0.025 and <0.45 µm) aluminium analysis. For measurement of dissolved aluminium, samples were

filtered using acid-washed 0.45 µm cellulose nitrate filters (25 mm, Sartorius Minisart, Goettingen, Germany) and 0.025 µm cellulose nitrate membrane filters (47 mm, Millipore, Darmstadt, Germany). Total aluminium samples include dissolved and precipitated forms, <0.45 µm samples include dissolved and some colloidal forms, <0.025 µm samples include dissolved and only low molecular-weight colloidal forms. Each filter was pre-washed with the solution to be filtered to reduce the effects of aluminium retention on the membrane due to the adsorption of charged species. Sub-samples were preserved with 5% and 2% HNO_3 (v/v) (Tracepur, Merck) for total and dissolved aluminium, respectively. A higher concentration of acid was required for the total aluminium sub-samples to digest and solubilize precipitates and algal cells that were part of the sample.

2.6.2 Metal analyses

Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to provide multi-element analysis of toxicity test solutions. Of particular interest were aluminium and copper, the toxicant of interest and reference toxicant, respectively.

Metal determinations were performed using an axially viewed ICP-AES (Varian 730-ES; Agilent Technologies 700, Australia) equipped with a short, wide-bore torch (High solids, 2.3 mm injector, Glass Expansion, Australia) and a nebuliser argon humidifier (Capricorn Humidifier with bypass, Glass Expansion, Australia) to prevent salt build-up on the ICP torch and plasma interface. Before use the instrument was allowed to "warm-up" for a minimum of 30 minutes and optimised against the 257.610 nm emission line with a 5 mg/L manganese solution.

Metal concentrations were calculated from a matrix-matched calibration curve. Calibration standards were prepared by dilution of certified multi-element stock solution (2007-LPC4R, QDC Analysts) with filtered seawater and preserved with 2% or 5% (v/v) HNO_3 (Tracepur, Merck) for dissolved and total metal determinations, respectively. A drift standard (calibration standard of 200 µg Al/L) was analysed once per 10 samples within an analytical run to assess and correct for drift that occurs when an instrument is operating. A rinse blank (Milli-Q containing 2% HNO3) was analysed after each drift standard to ensure this standard did not result in metal carry over into the following sample and for monitoring the baseline. The emission lines used, along with the limit of detection (LOD) are presented in Table 2.6. Values less than the LOD were substituted as half of the LOD for subsequent data analysis. All processing of analytical data for drift correction were performed using a Microsoft Excel program that assumed linear drift between each drift standard. A certified reference material for aluminium analysis in seawater was not commercially available so an in-house aluminium standard prepared from an independent standard was run with each batch of samples.

Table 2.6 ICP-AES emission lines and detection limits

Element	Emission line wavelength	LOD (3 x SD of the mean blank, n=4)
Al	167.019 nm (< 2 mg Al L^{-1})	0.3 ± 0.2 (n=54)
Al	176.502 nm (≥ 2 mg Al L^{-1})	40 ± 20 (n=22)
Cu	327.395 nm	0.2 ± 0.3 (n=8)

2.6.3 Quality assurance and control

Separate quality control experiments were performed to investigate potential sources of aluminium contamination in control flasks (see Appendix A.3 for more detail) and the loss of aluminium from test solutions due to adsorption to the glass flask in the absence of algae (see Appendix A.4 for more detail) over 72-h bioassays.

3 RESULTS

3.1 Aluminium speciation in bioassays

3.1.1 Solubility of aluminium in test solutions

The relationship between dissolved aluminium (<0.45 µm) and total aluminium concentrations in bioassay test solutions at the time of test initiation are shown in Figure 3.1. Concentrations of dissolved aluminium were equal to the total aluminium up to approximately 500 µg/L total aluminium, which is indicative of aluminium being predominately in the dissolved form below ~500 µg/L. Above this concentration both dissolved and precipitated forms of aluminium were present in the bioassay test solutions however, the proportion of dissolved aluminium decreased with increasing total aluminium, as shown in Figure 3.1, by the increasing divergence of dissolved aluminium from the y=x relationship with increasing total aluminium. For total aluminium concentrations in the range 500 – 10000 µg/L, dissolved aluminium reached a maximum of ~1500 µg/L at a total aluminium concentration of ~8000 µg/L. At all aluminium concentrations tested, there was a negligible difference between the <0.025 and <0.45 µm dissolved fractions, at both 0 h and 72 h, indicating an absence of colloids within the 0.025 to 0.45 µm size range (Figure 3.2).

3.1.2 Total aluminium in solutions over time

Figure 3.3 shows how total aluminium concentrations in the test solutions changed over the duration of bioassays (n=4) with *C. closterium*. Changes in total aluminium observed in bioassays with for *C. closterium* concurred with changes observed with *M. polymorphus* (n=3) and *P. tricornutum* (n=3) (Appendix B.1) and for this reason only results from bioassays with *C. closterium* are presented in Figure 3.3. Increases in total aluminium (mean ± S.D.) of 6 ± 1 µg/L (900 ± 500%, n=4) and 5 ± 3 µg/L (200 ± 100%, n=2), were observed in the control treatment (no added aluminium) and the lowest aluminium concentration treatment (10 µg/L), respectively. Quality control experiments investigating this contamination found that the increase in aluminium was not attributed to contamination by external ambient particulate aluminium (Appendix A.3). In brief, the contamination only occurred in treatments where algal cells were present and it is possible that interaction between the algal cells and the glass test flasks resulted in leaching of aluminium into solution, as aluminium is a major constituent of glass. However, the increase in dissolved aluminium was sufficiently low that it had negligible effect on higher exposure concentrations or algal growth rates.

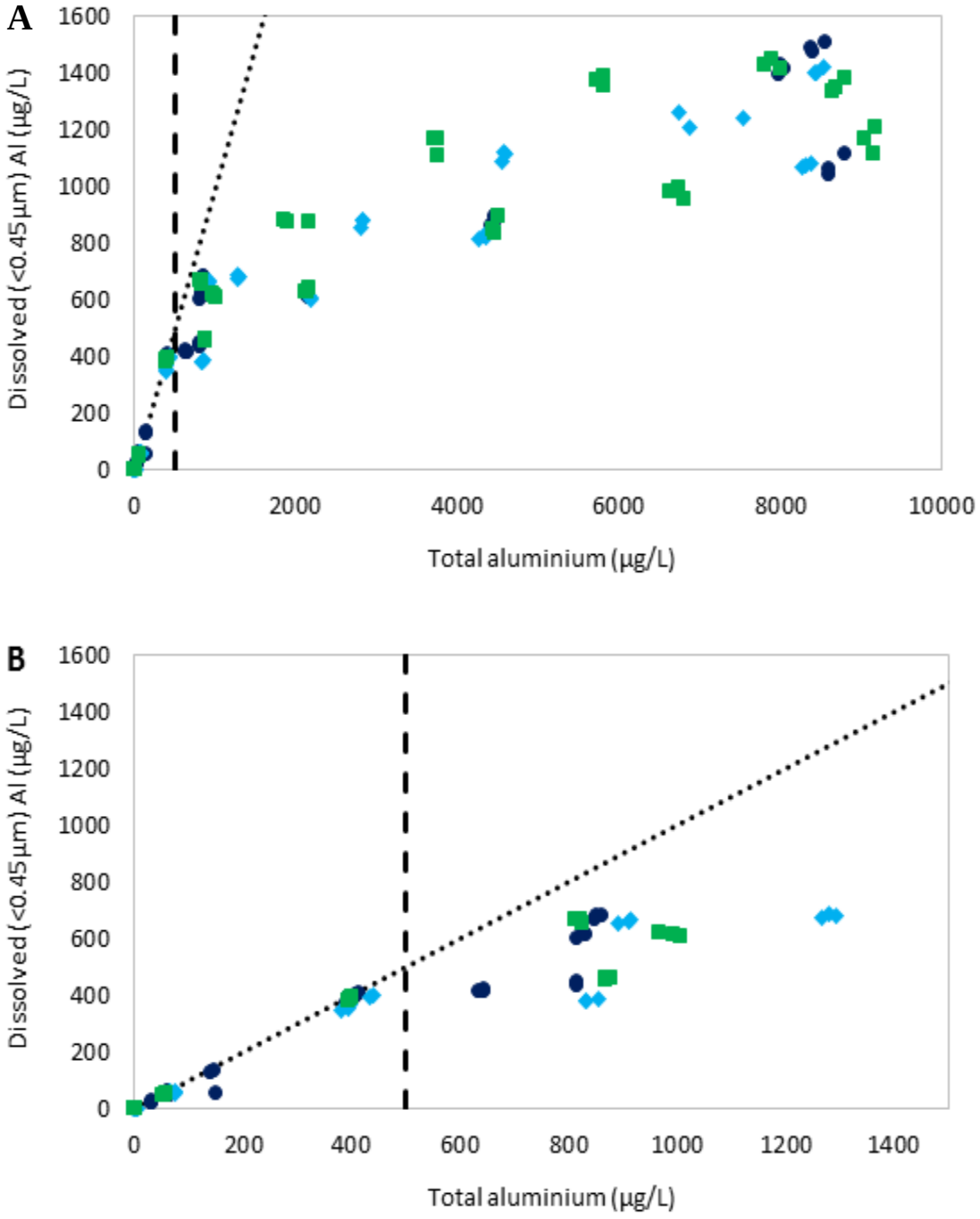


Figure 3.1 The relationship between dissolved (<0.45 µm) and total aluminium in bioassay test solutions at the time of test initiation (0 h) over the total concentration ranges: A) 0 to 10000 µg/L and B) 0 to 1500 µg/L. The black dotted line (y=x) represents the range of total aluminium concentrations for which dissolved aluminium is 100%. The black dashed line (x=500 µg Al/L) represents the approximate aluminium concentration above which precipitation begins. Bioassays were performed with three species of marine diatoms, and are represented by symbols (● = *Ceratoneis closterium*, ◆ = *Minutocellus polymorphus*, ■ = *Phaeodactylum tricornutum*). Each point represents a single replicate

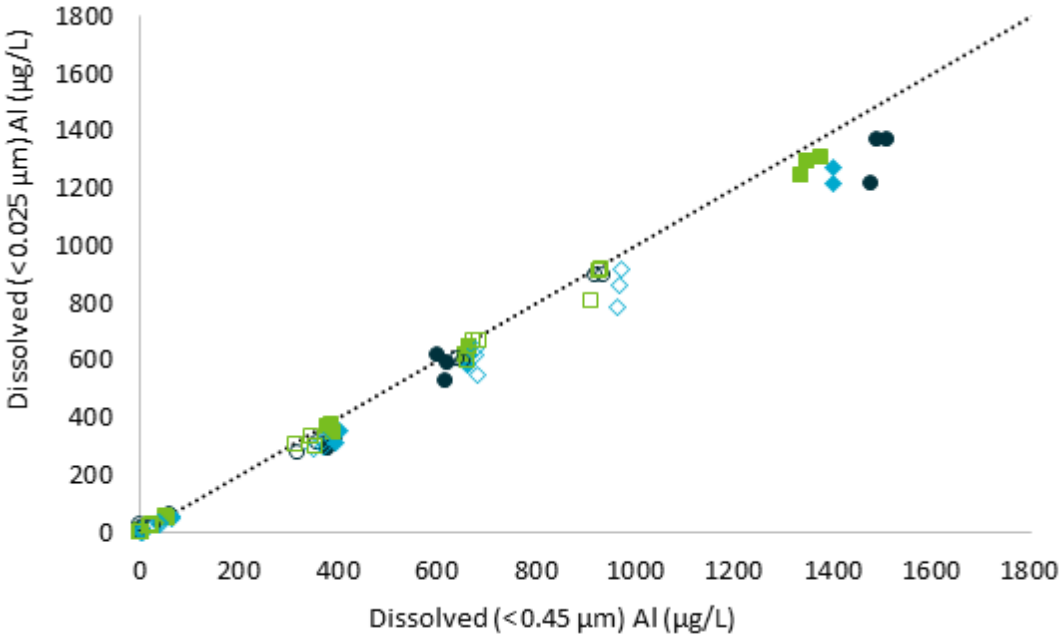


Figure 3.2 The relationship between dissolved (<0.025 and <0.45 µm) aluminium in bioassay solutions at the beginning (0 h; *closed symbols*) and end (72 h; *open circles*) of the bioassays. The black dotted line (y=x) represents the range of dissolved (<0.025 µm) aluminium concentrations for which dissolved (<0.45 µm) aluminium is 100%. Bioassays were performed with three species of marine diatoms, and are represented by symbols (● = *Ceratoneis closterium*, ◆ = *Minutocellus polymorphus*, ■ = *Phaeodactylum tricornutum*). Each point represents one individual replicate

Large decreases in total aluminium were observed between the nominal concentrations 50 to 200 µg/L. In tests exposing *C. closterium* to 50, 100 and 200 µg/L, the 72-h total aluminium concentration were 62 ± 0.4% (n=6), 50 ± 10% (n=12), 36 ± 7% (n=3) of concentrations at 0 h, respectively. Losses in total aluminium were also observed over 72 h in the absence of test organisms in the quality control experiments, with the greatest losses occurring within the first two hours of solutions being added to flasks and was attributed to adsorption of dissolved aluminium to flask glass walls (see Appendix A.4 for more details). Test concentrations closer to, and above, the solubility limit (500 µg/L) saw comparatively minor decreases in total aluminium. At higher exposures, 500, 750, 1000, 2500 and 5000 µg/L, the 72-h total aluminium concentrations were 12 ± 3% (n=6), 12 ± 2% (n=3), 7 ± 2% (n=9), 19 ± 1% (n=3), 17 ± 1% (n=3) of concentrations at 0 h, respectively. In contrast, at the highest aluminium concentration tested (10000 µg/L) total aluminium increased marginally over the 72-h bioassay by 5 ± 6% (n=9).

3.1.3 Dissolved aluminium in solutions over time

Figure 3.4 shows how dissolved (<0.45 µm) aluminium concentrations in the test solutions changed over the duration of bioassays (n=4) with *C. closterium*. Changes in dissolved (<0.45 µm) aluminium in bioassays with *C. closterium* concurred with changes with *M. polymorphus* (n=3) and *P. tricornutum* (n=3) (Appendix B.2) and for this reason only results from bioassays with *C. closterium* are presented in Figure 3.4. Similarly to total aluminium, decreases in dissolved (<0.45 µm) aluminium over the test duration were also observed below 200 µg/L with the greatest losses observed at the lower concentrations. In tests exposing *C. closterium* to 10, 50, 100 and 200 µg/L the 72-h dissolved aluminium concentration were 85 ± 10% (n =6), 99 ± 0.01% (n=3), 69 ± 14% (n=12), 41 ± 10% (n=3) of concentrations at 0 h, respectively. In the treatments where total aluminium was 500 µg/L, dissolved aluminium decreased only marginally: 10 ± 5% (n=6). Between 750 and 5000 µg/L total aluminium the dissolved aluminium concentrations increased over the first 24 h, and remained stable at that value over the test duration. In treatments exposing *C. closterium* to 10000 µg/L total aluminium, loss of dissolved aluminium was observed. In contrast to what was observed for 10000 µg/L total aluminium, the 72-h dissolved aluminium concentration was 34 ± 6% (n=9) of the concentration at 0 h.

3.2 Diatom growth-rate inhibition bioassays

3.2.1 Test acceptability

For all tests, diatom control growth rates were acceptable (Stauber et al., 2005), with ≥ 1 doublings per day indicating healthy cells and the percentage coefficient of variation < 10% (Table 3.1). Changes in pH were minimal across all treatments (controls and metal exposures), with initial pH values of 8.2 ± 0.05 (n=201), and changes not exceeding 0.3 pH units by the end of the test. Copper reference tests demonstrated that the diatom cultures exhibited similar sensitivity throughout the project (Appendix A.1), and were consistent with previous copper exposure assays (Levy et al., 2007). Further test validation was performed as the standard protocol test volume was increased in this study from 50 mL to 100 mL to accommodate the extra volume of sample required for metal speciation (<0.025 µm, <0.45 µm and total) analysis. Bioassays with the two treatment volumes were performed concurrently, and showed that control growth rates and sensitivity to aluminium between the two treatment volumes concurred. This indicated that volume had no influence on the effect of aluminium exposure to the diatoms (Appendix A.2).

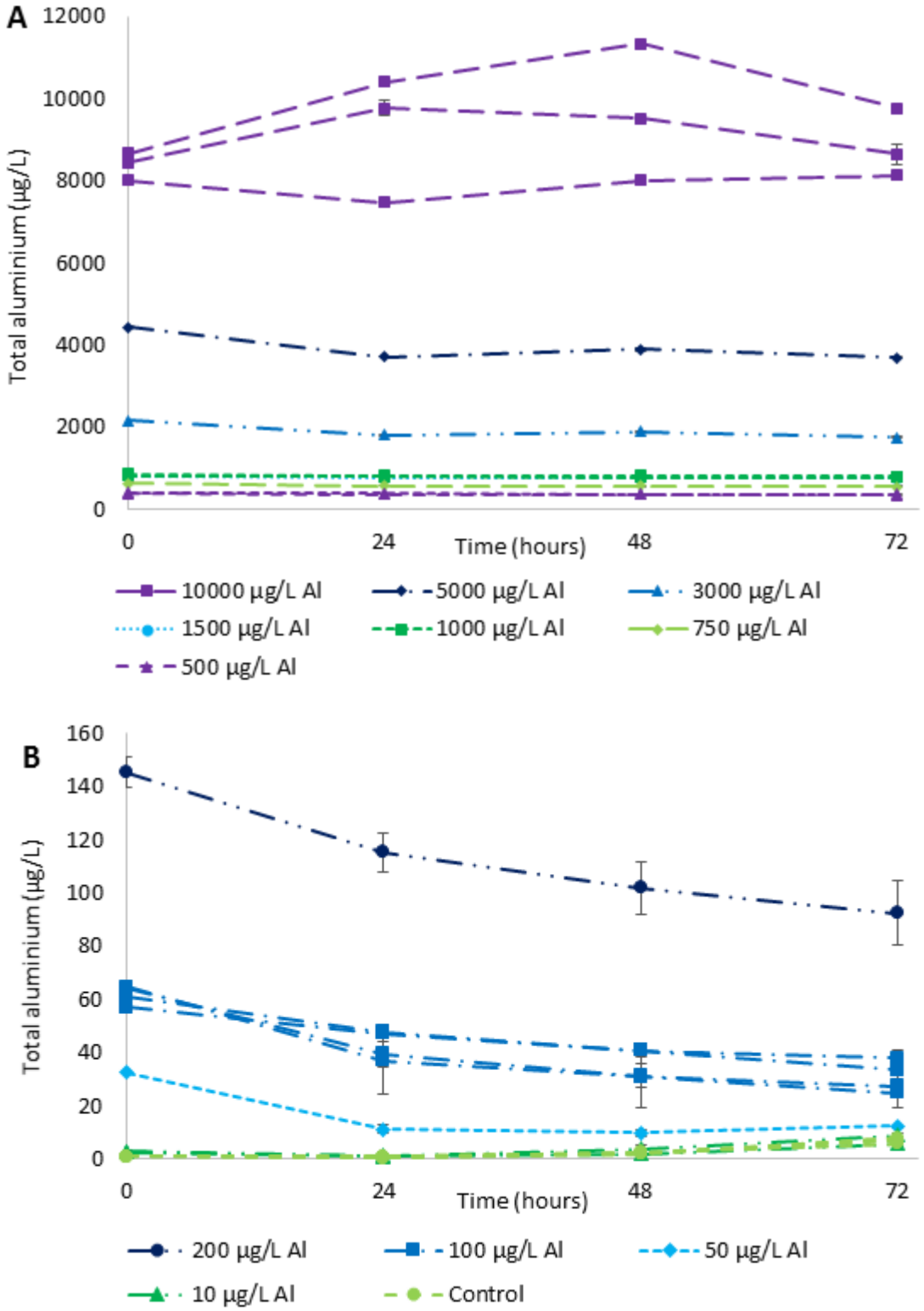


Figure 3.3 The concentration of total aluminium in bioassay test solutions with *Ceratoneis closterium* as a function of time for nominal aluminium concentrations: A) 500 to 10000 µg/L and B) 0 to 200 µg/L (mean ± standard deviation, n=3, vertical error bars). Concentrations of 10, 100, 500, and 10000 µg/L were tested in more than 1 bioassay

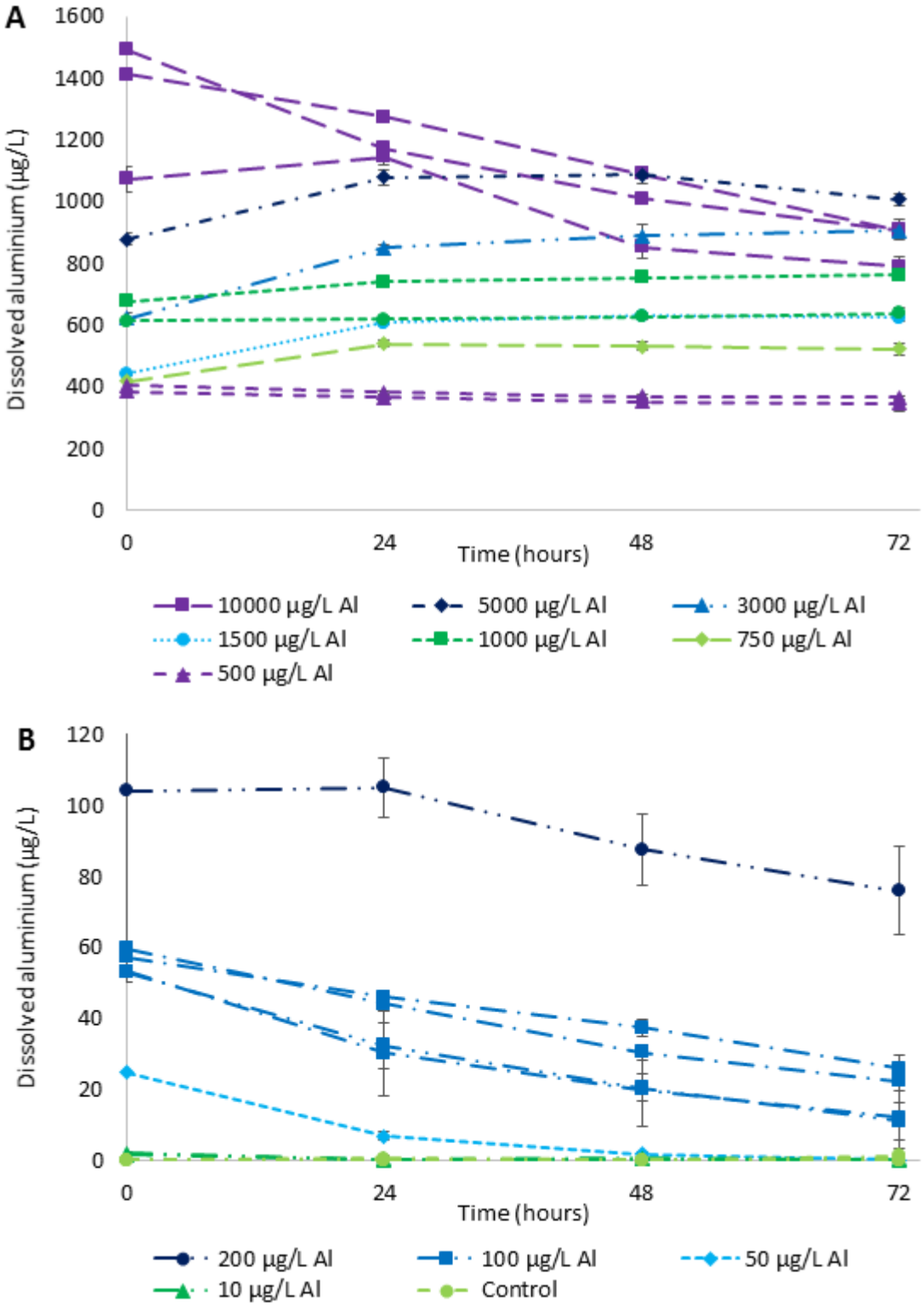


Figure 3.4 The concentration of dissolved aluminium (<0.45 µm) in bioassay test solutions with *Ceratoneis closterium* as a function of time for nominal aluminium concentrations: A) 500 to 10000 µg/L and B) 0 to 200 µg/L (mean ± standard deviation, n=3, vertical error bars). Concentrations of 10, 100, 500, and 10000 µg/L were tested in more than 1 bioassay

Table 3.1 Marine diatom control growth rates and percent coefficient of variation over 72-h growth-rate inhibition bioassays

Marine diatom Species	Control performance	
	Growth rate [a]	% CV [b]
Ceratoneis closterium[c]	1.9 ± 0.14	≤ 6
Minutocellus polymorphus[d]	2.1 ± 0.14	≤ 3
Phaeodactylum tricornutum[d]	1.5 ± 0.094	≤ 5

[a] Control growth rate in doublings/day

[b] %CV: percent coefficient of variation of control growth rate

[c] Four separate bioassays conducted and results pooled

[d] Three separate bioassays conducted and results pooled

3.2.2 Concentration-response curves and toxicity values

The sensitivities of the three marine diatoms to aluminium over 72 h are shown in Figure 3.5. The response of each diatom is plotted against both total and dissolved (<0.45 µm) aluminium concentrations at the time of test initiation to compare the relative importance of each form in eliciting toxicity. The response of each diatom, in relation to both total and dissolved (<0.45 µm) aluminium at the beginning, end and using time-averaged aluminium concentrations presented on a linear scale are also provided in Appendix B.3 for comparison. From this data IC10 and IC50 values were calculated using both initial total and dissolved (<0.45 µm) aluminium concentrations and time-averaged total and dissolved (<0.45 µm) aluminium concentrations to account for changes in aluminium concentrations over the duration of the bioassays (Table 3.2). For each diatom, the results are the combination of 3 (or 4 in the case of *C. closterium*) separate bioassays pooled together, using the slope of the $\log_{10}$ transformed cell density over 72 h for each test.

The concentration-response curve of *C. closterium* differed markedly from that of the other two species with the decline in growth rate occurring over 3 orders of magnitude (Figure 3.5). A slight stimulation in growth of *C. closterium* occurred at aluminium concentrations below 60 µg/L in some tests. Concentrations above 60 µg/L caused a significant decrease in growth however, complete inhibition was not observed, with a maximum of 80% inhibition occurring at the highest aluminum treatment tested (10000 µg/L). The curves plotted with initial total and dissolved (<0.45 µm) aluminium concentrations were in accord up to about 1000 µg/L.

The onset of declining growth rate occurred at much higher aluminium concentrations for *M. polymorphus* and *P. tricornutum* and the concentration range was much narrower (spanning only one

order of magnitude) compared to *C. closterium* (Figure 3.5). Increasing concentrations of aluminium did not appear to affect the growth of *M. polymorphus* below 300 µg/L, above this however, there was a sharp decline in growth rate (Figure 3.5 B). The curves for total and dissolved (<0.45 µm) aluminium concentrations followed the same shape but deviated above 500 µg/L due to the onset of precipitation, decreasing the proportion of dissolved aluminium in the total aluminium pool.

Increasing concentration of aluminium did not appear to affect the growth of *P. tricornutum* below 500 µg/L (Figure 3.5). Above this there were large differences between the total and dissolved (<0.45 µm) aluminium growth-rate curves. Growth rate reached 60% of the control where dissolved aluminium reached a maximum of ~1000µg/L and total aluminium exceeded ~4000 µg/L. This region of the concentration-response curve was dominated by precipitated aluminium and there was little change in dissolved aluminium concentration with increasing total aluminium concentration. Growth-rate declined further to 20% of the control when total aluminium reached ~9000 µg/L.

Based on the 10% inhibition of population growth rate values (IC10) calculated from the pooled data set *C. closterium,* was the most sensitive to aluminium with the lowest 72-h IC10 (Table 3.2). *M. polymorphus* displayed moderate sensitivity to aluminium with the next lowest 72-h IC10, while *P. tricornutum* displayed the greatest aluminium tolerance with the highest 72-h IC10 value observed (Table 3.2). IC10 and IC50 concentrations based on dissolved (<0.45 µm) aluminium concentration were lower than those based on total aluminium concentrations for all species with greater deviation between the endpoints calculated with dissolved (<0.45 µm) and total aluminium for the more tolerant species investigated (Table 3.2). IC10 and IC50 values based on measured time averaged aluminium concentrations for *C. closterium* were lower than those based on initial measured aluminum concentrations whereas, for *M. polymorphus* and *P. tricornutum* values were in good agreement (Table 3.2). The two diatoms with the siliceous cell wall (*C. closterium* and *M. polymorphus*) were more sensitive to aluminium than *P. tricornutum,* the only species with a weakly siliceous cell wall. No pattern was observed according to cell shape or cell size with both the most sensitive and the most tolerate species being the larger pennate diatoms.

3.2.3 Influence of initial cell density on diatom sensitivity to aluminium

For all cell density tests, diatom control growth rates were once again acceptable, with ≥ 1 doublings per day (Table 3.3). Significantly higher ($p < 0.05$) control growth rates were observed at lower initial cell densities for all diatoms. The percent coefficient of *P. tricornutum* was slightly higher than the expected 10%, due to greater growth observed in one replicate. All control and metal exposure treatments had similar initial pH values (8.2 ± 0.05), and changes did not exceed 0.4 pH units by the end of the test.

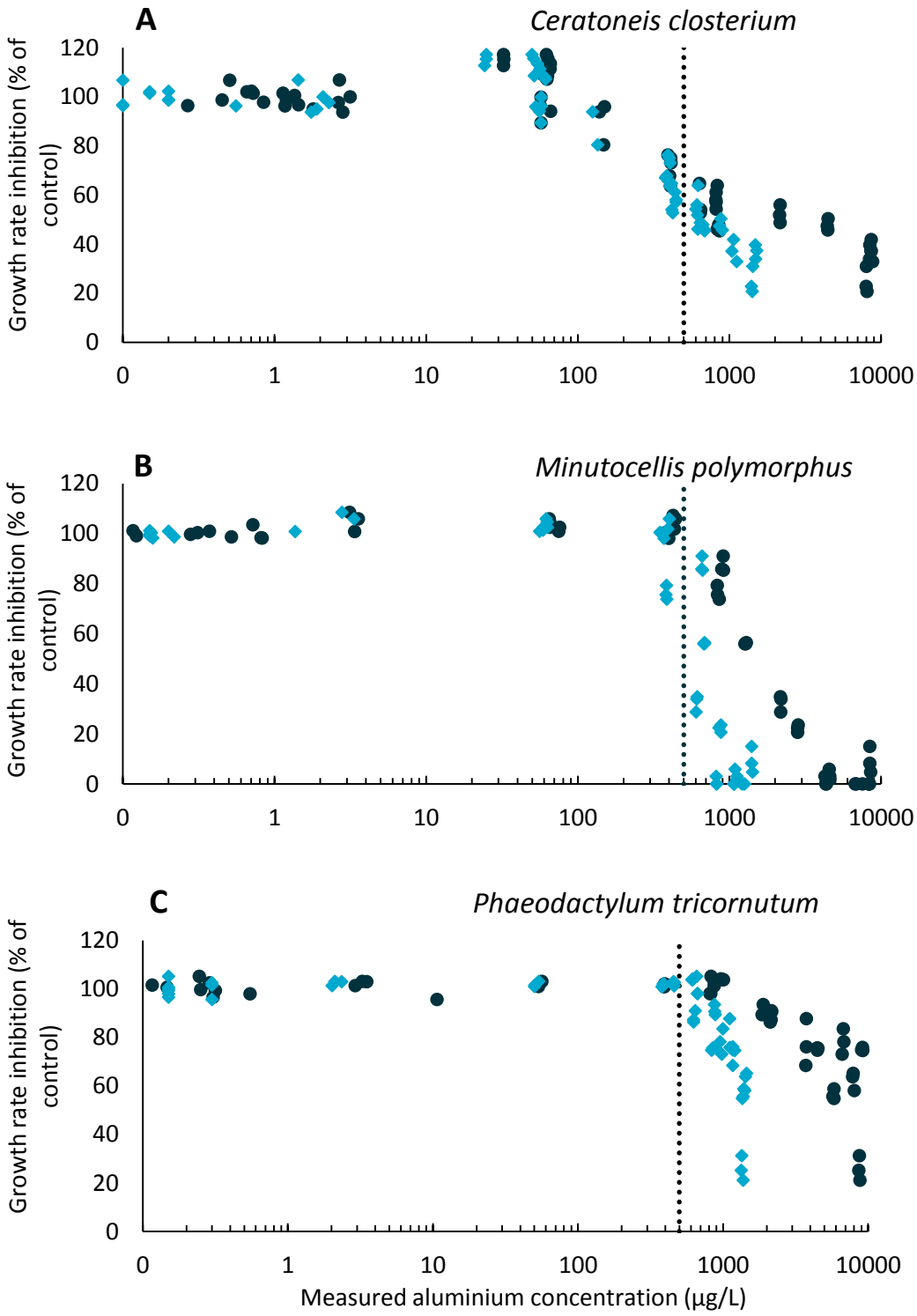


Figure 3.5 The effect of aluminium on the growth rate (% of control) of three marine diatoms after 72-h exposure (A) *Ceratoneis closterium*, (B) *Minutocellus polymorphus* and (C) *Phaeodactylum tricornutum*. Points represent pooled data from ≥ 3 separate bioassays. Initial (0-h) total (*circles*) and dissolved (*diamonds*) aluminium concentrations. The black dotted line (x=500 µg Al/L) represents the approximate aluminium concentration above which precipitation begins

Table 3.2 Sensitivity of marine diatoms to aluminium in 72-h growth-rate inhibition bioassays

Diatom Species	72-h Growth rate inhibition (µg/L)			
	Total aluminium[a]		Dissolved aluminium[b]	
	IC10[c] (95% CL)	IC50[c] (95% CL)	IC10 (95% CL)	IC50 (95% CL)
Using initial exposure data (0 h), 72-h growth-rate inhibition bioassays				
Ceratoneis closterium[d]	100 (44-140)	840 (810-850)	81 (61-98)	620 (300-630)[f]
Minutocellus polymorphus[e]	530 (470-590)	1360 (1300-1400)	370 (360-390)	692 (690-700)[f]
Phaeodactylum tricornutum[e]	2000 (1200-2600)	> 9100 (NC)	740 (650-780)[f]	1300 (1300-1340)[f]
Using time-averaged aluminium concentrations. 72-h growth-rate inhibition bioassays				
Ceratoneis closterium[d]	80 (55-100)	807 (780-810)	69 (44-89)	680 (570-710)[f]
Minutocellus polymorphus[e]	540 (460-600)	1370 (1300-1400)	440 (420-470)	778.5 (778.2-778.8)[f]
Phaeodactylum tricornutum[e]	2100 (2000-2200)	> 9500 (NC)	920 (910-940)[f]	>1200 (NC)[f]

[a] Toxicity endpoints calculated from measured total aluminium concentrations

[b] Toxicity endpoints calculated from measured dissolved (<0.45 µm) aluminium concentrations

[c] IC10/IC50: Concentration of aluminium to cause a 10% and 50% inhibition of the diatom cell growth rate over 72-h respectively

[d] Four separate bioassays conducted and results pooled

[e] Three separate bioassays conducted and results pooled

[f] Dissolved effect concentrations exceed the 500 µg/L total aluminium onset of precipitation therefore they represent a combination of dissolved and precipitated aluminium i.e. the effect is not due to dissolved aluminium only

NC = not calculable

Table 3.3 Effect of initial cell density on marine diatom control growth rates and percent coefficient of variation over 72-h growth-rate inhibition bioassays

Initial cell density (cells/mL)	*Ceratoneis closterium*		*Minutocellus polymorphus*		*Phaeodactylum tricornutum*	
	Growth rate[a]	% CV[b]	Growth rate	% CV	Growth rate	% CV
10^3	1.93* ± 0.08	4	1.75* ± 0.03	1	1.25* ± 0.03	2
10^4	1.59* ± 0.03	2	1.29* ± 0.02	2	1.19* ± 0.01	1
10^5	NM	NM	1.0* ± 0.1	11	1.02* ± 0.05	5

[a] Control growth rate in doublings/day

[b] %CV: percent coefficient of variation of control growth rate

NM = not measured

* = Significant difference ($p < 0.05$)

Growth rate inhibition of the three marine diatoms exposed to aluminium for 72 h at initial cell densities ranging from 10^3 to 10^5 cells/mL are shown in Figure 3.6. For both *C. closterium* and *M. polymorphus,* as the initial cell density increased, the toxicity to aluminium also increased. There was no consistent relationship between initial cell density and aluminium toxicity for *P. tricornutum*. For *C. closterium,* a significant decrease ($p < 0.05$) was observed in the growth rate value between the initial cell densities of 10^3 and 10^4. For *M. polymorphus*, no significant difference ($p < 0.05$) was observed between the initial cell densities of 10^3 and 10^4, but there was however a significant decrease ($p < 0.05$) between these two treatments and the 10^5 treatment. For *P. tricornutum* no significant difference was observed between the 10^3 and 10^4 treatment or the 10^3 and 10^5 treatment; there was however a significant increase ($p < 0.05$) between the 10^4 and 10^5 treatment.

The form of aluminium in solution was determined to identify if the algae themselves were having an effect on the chemical speciation, such as by exuding extracellular material that complexes aluminium. As noted before, with the initial cell density of 10^3 cells/mL, there was a negligible difference between the dissolved size fractions (<0.025 and <0.45 µm) at aluminium concentrations up to 10000 µg/L total aluminium for all algal species (Figure 3.2). Results from this experiment revealed that at 10^5 cells/mL there was also a negligible difference (6%) between the dissolved size fractions (<0.025 µm and <0.45 µm) for *M. polymorphus* at 1750 µg/L total aluminium (Table 3.4). For *P. tricornutum* however, a 32% decrease in dissolved aluminium (<0.025 µm) occurred relative to the <0.45 µm size fraction (Table 3.4). *P. tricornutum* showed greater tolerance to aluminium at the initial cell density of 10^5 cells/mL.

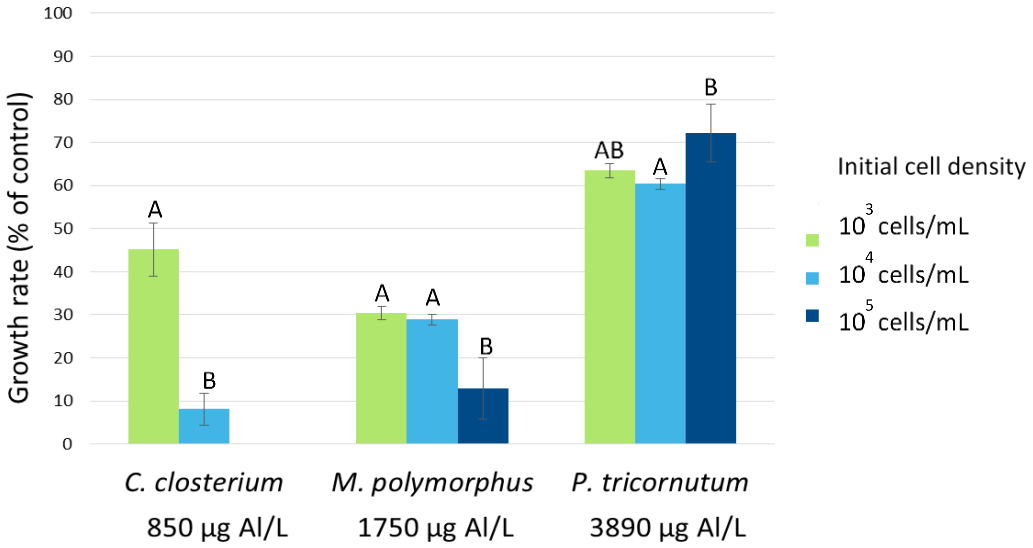


Figure 3.6 Effect of initial cell density on the sensitivity of 3 marine diatoms to aluminium. Exposure concentrations were close to the individual IC50 values for *Ceratoneis closterium* and *Minutocellus polymorphus* and the IC25 value for *Phaeodactylum tricornutum*. Different letters indicate statistical differences in response within species ($p \leq 0.05$). Each column represents the mean of 3 replicates ± the standard deviation (*vertical error bars*).

Table 3.4 Comparison of <0.025 µm and <0.45 µm filtered size fractions of measured dissolved aluminium (at 72 h) in bioassay test solutions with an initial algal cell density of 10^5 cells/mL

Initial cell density (cells/mL)	*Minutocellus polymorphus* Dissolved Al (nominal 2000 µg/L)			*Phaeodactylum tricornutum* Dissolved Al (nominal 5000 µg/L)		
	<0.025	<0.45	%difference	<0.025	<0.45	%difference
10^5	890 ± 50	950 ± 30	6%	560 ± 80	800 ± 100	32%

3.3 Method development: diatom membrane permeability

3.3.1 Optimisation of diatom cell staining with SYTOX Green

Preliminary experiments were performed to determine the optimum staining concentration of SYTOX Green and incubation time for the three marine diatoms. The evaluation of plasma membrane permeability was based on the uptake of SYTOX Green dye by cells with a permeabilized plasma membrane and the subsequent enhancement of green fluorescence (FL1) upon binding with nucleic acid and excitation with a 450-490 nm light source (Sato et al. 2004).

The intensity of green fluorescence of the positive control cells (heat-treated) increased with increasing SYTOX Green concentration while the fluorescence of the negative control cells (healthy cells) remained very low allowing for the two populations of cells to be easily discriminated even at low SYTOX Green staining concentrations (Figure 3.7 A). With the aim of obtaining a strong and even fluorescence signal and clear separation between the two populations at a low dye concentration (to decrease the costs associated with using the fluorochrome) the staining concentration of 0.5 µM SYTOX Green was selected as the optimal staining condition. All diatom species obtained a high level of fluorescence intensity within 5 min and remained stable until 60 min, the maximum time point measured (Figure 3.7 B). With the aim of achieving a strong green fluorescence signal with a short incubation time (to keep the time associated with processing sample to a minimum) an incubation time of 5 min was selected as the optimal staining condition. The final staining concentration of 0.5 µM SYTOX Green and incubation for 5 min in the dark were suitable for all three marine diatoms (representative flow cytometric images showing clear separation between auto green fluorescence and enhanced SYTOX Green fluoresce of cells with permeabilized membranes are provided in Appendix B.4). These conditions were therefore used for all subsequent flow cytometry experiments.

3.3.2 Validation of flow cytometry results with confocal microscopy

The validation of using the fluorochrome SYTOX Green for evaluating plasma membrane permeability of the three marine diatoms by flow cytometry (Figure 3.6) under the optimum staining conditions was performed with confocal laser-scanning microscopy. All cells were stained using the optimized staining conditions selected above (incubation for 5 min with 0.5 µM SYTOX Green).

SYTOX Green was completely excluded from the negative control cells (healthy cells) of *C. closterium* – the cells remained unstained (Figure 3.8 A). Whereas SYTOX Green penetrated the plasma membrane of the positive control cells (heat-treated cells) of *C. closterium* indicated by the bright fluorescence signal observed (Figure 3.8 B). Confirmation that SYTOX Green also penetrated the plasma membrane of the positive control cells (heat-treated cells) of *P. tricornutum* is demonstrated in Figure 3.8 C. Confirmation for *M. polymorphus* was not possible due to its small size and reaching the limit of magnification of the confocal microscope.

The large difference in green fluorescence intensity allowed a clear distinction between cells with an intact plasma membrane and cells with a permeabilized plasma membrane to be observed further confirming the suitability of the optimized staining conditions (Figure 3.8 A and B).

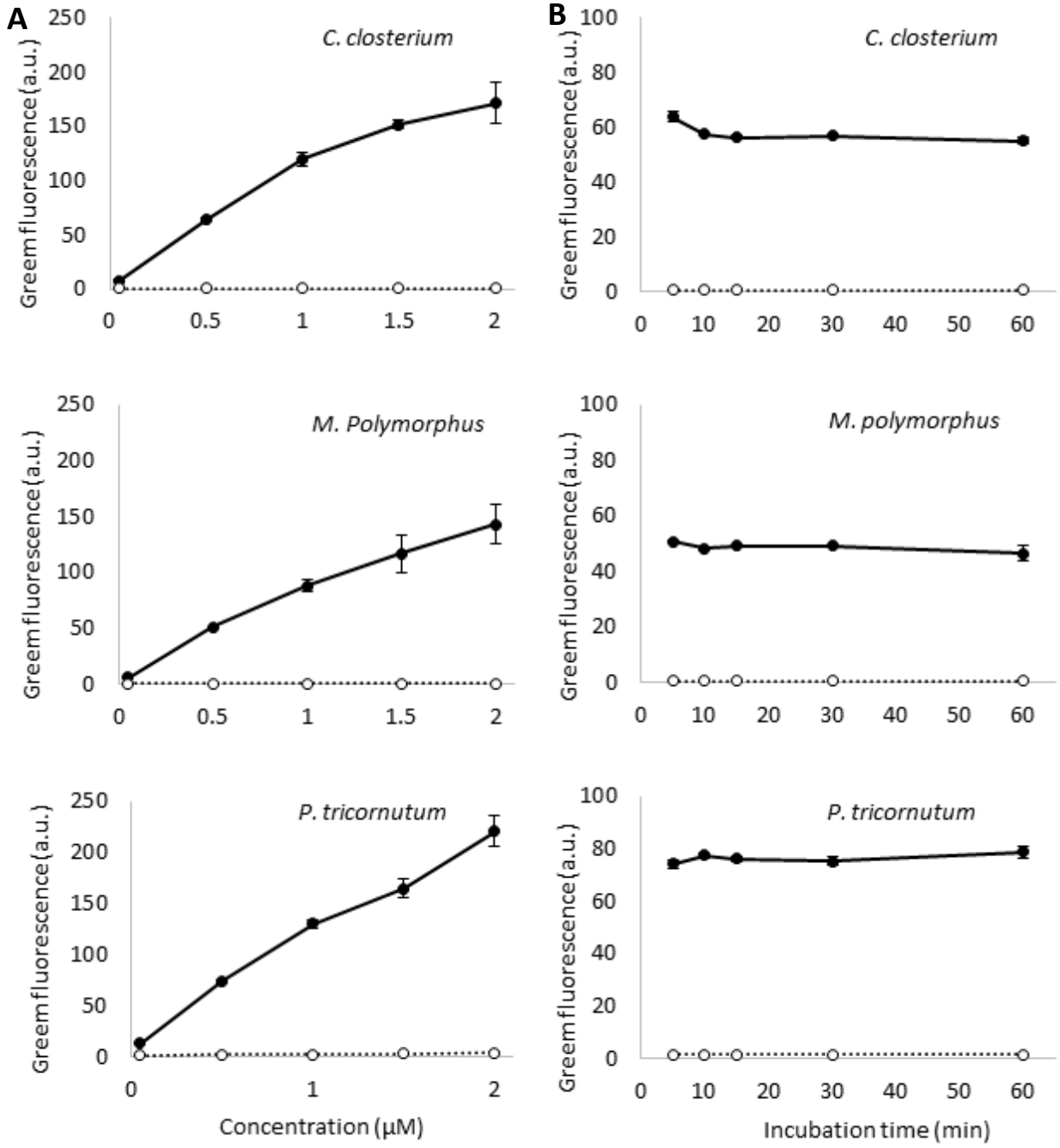


Figure 3.7 Optimisation of SYTOX Green staining conditions for cultures of *Ceratoneis closterium*, *Minutocellus polymorphus* and *Phaeodactylum tricornutum*. A) optimisation of SYTOX Green staining concentration (µM) while maintaining incubation time at 5 min, and B) optimisation of incubation time (min) with SYTOX Green (0.5 µM). Cultures consisted of a 1:1 mixture of negative control cells (healthy cells) (*open circles*) and positive control cells (heat-treated at 60°C for 5 min) (*closed circles*). Each point represents the mean of 2 replicates ± the standard deviation (*vertical error bars*).

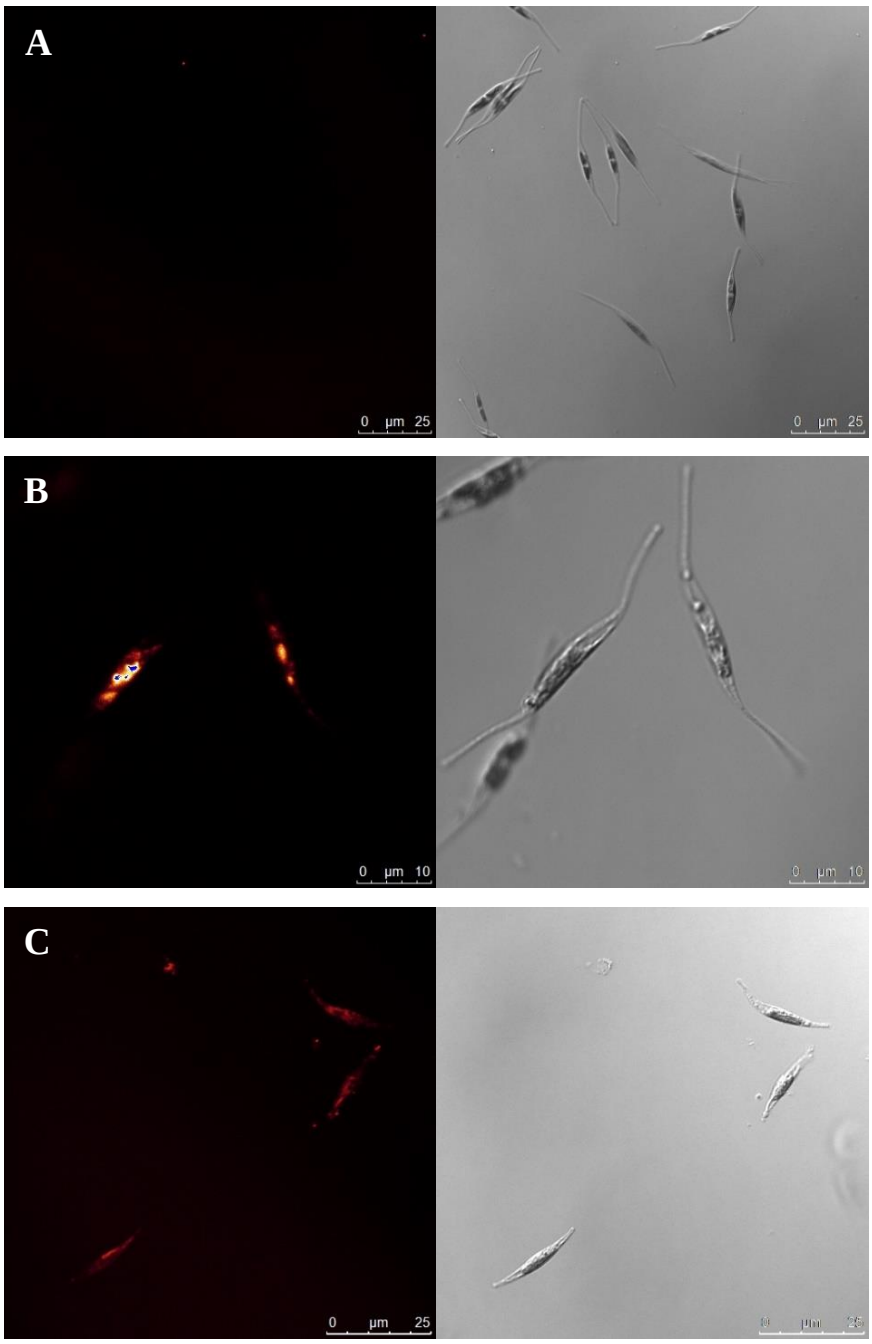


Figure 3.8 Visualisation of diatom cultures stained with SYTOX Green using Confocal laser-scanning microscopy (A) negative control cells (healthy cells) of *Ceratoneis closterium*, (B) positive control cells (heat-treated cells) of *C. closterium*, and (C) positive control cells of *Phaeodactylum tricornutum*. Cells were incubated with 0.5 µM SYTOX Green for 5 min in the dark. Positive control cells underwent heat-treatment in a 60°C water bath for 5 min. Healthy cells are not stained (A). Cells with a permeabilized cell membrane displayed fluorescence (B and C). Images in the left column are fluorescence images; fluorescence was collected between 500 and 590 nm (green fluorescence shown red in these images). Images in the right column are phase contrast images of the same cells in the fluorescence images

3.4 Aluminium effects on diatom membrane permeability

Table 3.5 shows the effect of aluminium on plasma membrane permeability of *C. closterium*, *M. polymorphus* and *P. tricornutum* after 24 h of exposure. For all diatoms, cells in the positive control treatment showed > 99% of the population had a permeabilized plasma membrane and in the healthy control treatment < 5%, indicating the SYTOX Green stock solution was responding appropriately. For *C. closterium* the greatest proportion of the population with a permeabilized plasma membranes was observed at the 1000 µg/L treatment with 7.5%; for all other treatments the proportion of the population with permeabilized membranes was less than 4% in line with no aluminium exposure (the control). For *M. polymorphus*, the proportion of the population with a permeabilized plasma membrane for aluminium treatments up to 2500 µg/L was ≤ 1%; but increased slightly in the 5000 and 10000 µg/L treatments with values of 6 and 10% observed, respectively. For *P. tricoruntum* the proportion of the population with a permeabilized plasma membrane remained low in all treatments with the greatest value of 5% observed for the 10000 µg/L treatment.

Inspection of the flow cytometric dot plots of the treatments which displayed the greatest proportion of cells with permeabilized plasma membranes reveals the increase to be attributed to the spread of the population around the healthy population increasing and spreading over into the E2 region, rather than appearing in the E2 region where cells with a permeablized membrane would appear (Appendix B.5). The 1000 µg/L aluminium treatment for *C. closterium* was viewed with confocal microscope and confirmed the cells did not have a permeabilized plasma membrane (Figure 3.9). Therefore, the flow cytometric data indicates that aluminium toxicity did not increase permeability of the plasma membrane.

Results from 48- and 72-h exposures (Appendix B.6) concurred with those at 24 h, except for a slight increase in percent membrane permeability at concentrations greater than 500 g/L. At these higher metal concentrations there are lower cell densities which means that scatter of data caused larger percentage shifts into the SYTOX Green region and is not indicative of an increase in percent membrane permeability.

Table 3.5 Membrane permeability of marine diatoms exposed to aluminium for 24 h measured following incubation with 0.5 µM SYTOX Green for 5 min.

Nominal Al treatment (µg/L)	Membrane permeability (% of cells in E2)[a]		
	Ceratoneis closterium	*Minutocellus polymorphus*	*Phaeodactylum. tricornutum*
Positive control[b]	99.6 ± 0.4	99.7 ± 0.4	99.6 ± 0.3
Control	4.6 ± 1.5	0.4 ± 0.2	1.2 ± 0.5
100	2.2 ± 0.5	0.1 ± 0.1	0.6 ± 0.1
500	3.9 ± 1.7	0.2 ± 0.3	0.3 ± 0.1
1000	7.5 ± 2.0	0.2 ± 0.2	1.3 ± 0.5
2500	3.4 ± 1.2	1.0 ± 0.7	3.1 ± 0.6
5000	3.5 ± 1.0	6.2 ± 1.3	3.8 ± 1.5
10000	3.8 ± 2.1`	10.5 ± 3.2	4.7 ± 2.0

[a] Mean ± SD (n ≥ 3)

[b] Cells heat treated at 60°C for 5 min to permeabilize the plasma membrane

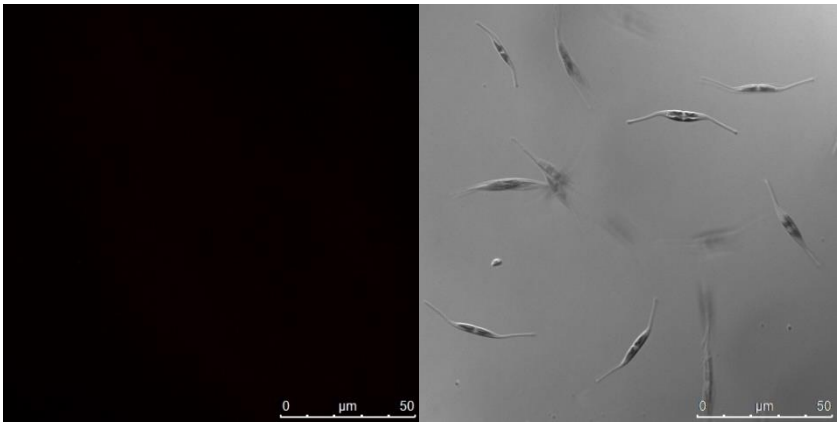


Figure 3.9 Visualisation of *Ceratoneis closterium* exposed to 1000 µg/L aluminium for 24 h stained with SYTOX Green (0.5 µM for 5 min) using confocal laser-scanning microscopy. No SYTOX Green fluorescence was observed indicating intact plasma membranes. Fluorescence image on the left (fluorescence was collected between 500 and 590 nm) while image on the right is a phase contrast image taken of the same cells in the fluorescence image

4 DISCUSSION

4.1 Aluminium speciation in bioassays

4.1.1 Solubility of aluminium in test solutions

Investigation of the solubility of aluminium in test solutions in the presence of diatoms from this study are consistent with those of Angel et al. (2014) which also showed dissolved aluminium was equal to the total aluminium up to a limit of 500 µg/L in seawater in the absence of test organisms. At higher aluminium test concentrations (500 to 10000 µg/L total aluminium) the current study found a maximum dissolved aluminium concentration of 1500 µg/L, slightly greater than that observed by Angel et al. (2014) of 1250 µg/L. Golding et al. (2014) examined solubility of aluminium in bioassays with a suite of different marine microalgae and invertebrates and also found that aluminium solubility increased in the presence of biota with a maximum dissolved aluminium concentration of 1700 µg/L observed at higher test concentrations (up to 100000 µg/L). In addition, the onset of aluminium precipitation increased from 500 µg/L to 1000 µg/L when a range of different marine biota were present (Golding et al., 2014). A closer inspection of the Golding et al. (2014) data revealed that there was variation in the dissolved versus total aluminium relationship between 500 µg/L and 1000 µg/L total aluminium suggesting that 500 µg/L total aluminium still represented a threshold for the onset of precipitation which became more clearly define above 1000 µg/L.

The form of dissolved aluminium in the bioassay solutions was further investigated in the current study by comparing the two dissolved aluminium size fractions <0.025 and <0.45 µm measured at 0 and 72 h. Results revealed that there was negligible difference between the <0.025 and <0.45 µm dissolved fractions at either 0 h or following growth after 72 h, indicating that negligible colloidal aluminium within the 0.025 to 0.45 µm size range formed in the presence of microalgae. This indicates that dissolved aluminium was predominantly present as truly soluble species and low molecular weight forms of aluminium. Furthermore this indicates that any biogenic exudates formed in bioassays conducted with an initial cell density of 10^3 cells/mL and aluminium concentrations up to 10000 µg/L did not adsorb or complex aluminium.

In the current study the response of marine diatoms to aluminium exposure was determined for concentrations in the range of 10 to 10000 µg/L. The implication of the investigations into the solubility of aluminium in bioassay solutions of the current study is that the marine diatoms were

exposed to the anionic and neutral species of dissolved aluminium below 500 µg/L, and a combination of both dissolved and precipitated species above 500 µg/L.

4.1.2 Changes to aluminium speciation over time

Investigations of the concentrations of total and dissolved (<0.45 µm) aluminium in solution over the course of the bioassay from the current study revealed three major trends: 1) loss of both total and dissolved (<0.45 µm) aluminium below 500 µg/L total aluminium; 2) increase of dissolved aluminium in the first 24 hours with total aluminium concentrations remaining stable, between 500 and 5000 µg/L total aluminium; and 3) large losses of dissolved (<0.45 µm) aluminium over the bioassay duration in the highest aluminium concentration tested (10000 µg/L) with variable total aluminium. The pH of the test solutions were measured at the beginning and end of the bioassays and did not change by more than 0.3 pH units by the end of the test, thus it is unlikely that the changes in speciation observed were due to changes in pH. Typically small increases in pH are observed as a result of photosynthetic activity and depletion of carbon dioxide which would, if anything, mean more aluminium would be in the aluminate form rather than the precipitated aluminium hydroxide form.

Losses of dissolved aluminium are likely to be the result of either adsorption of dissolved aluminium to container walls, algal cell surfaces or to precipitated aluminium, most likely $Al(OH)_3$. (Angel et al., 2014). The fact that decreases in both dissolved (<0.45 µm) and total aluminium occurred below 500 µg/L supports dissolved aluminium being lost to the glass container walls. This finding seems to be consistent with those of previous studies that have examined copper concentrations over the course of bioassays with microalgae. In toxicity tests exposing two green freshwater microalgae species to copper, Franklin et al. (2002), found that for both species 50-60% of copper was still present in solution as dissolved copper, 4-17% represented the intra- and extra-cellular copper fraction depending on the copper concentration used and approximately 20% had adsorbed to flask walls despite silanisation of the glass flasks prior to the bioassay being performed. Silanisation of test flasks is performed to minimize the loss of metal to the glass wall, but as evidence in the current study and the study of Franklin et al. (2002) adsorptive losses to the glass flask walls still occur.

A review of the use of alternative test containers for algal bioassays by Stauber and Davies (2000) found that substitution of glass flasks with plastic (e.g. Teflon, polycarbonate, polyethylene or polystyrene) is not recommended as it may lead to reduced algal growth rates because of reduced light penetration (in the case of Teflon), release of inhibitory chemicals used in plastic manufacture and sterilization, or to the inability of benthic algae to attach to these surfaces. Their review also looked at the impact of pre-conditioning glass surfaces prior to toxicity testing using copper and the

marine alga *C. closterium*. After preconditioning for 2 or 24 h, the mass of copper bound to the glass was double that of copper on the glass with no preconditioning, suggesting that preconditioning was of no use in reducing adsorptive losses (Stauber and Davies, 2000). The use of glass test flasks are still the preferred vessel for performing bioassays but only after thorough investigations of metal losses by adsorption to the container surface are undertaken (Stauber and Davies, 2000).

The increase in dissolved aluminum over the first 24 h observed between 500 and 5000 µg/L while total aluminium remained constant suggests that the conditions were dynamic. Lastly the large loss of dissolved aluminum at 10000 µg/L suggests that dissolved aluminium undergoes adsorption onto amorphous $Al(OH)_3$. These observation are consistent with the findings of Angel et al. (2014) which showed that above the solubility limit, dissolved aluminium concentrations increased above the solubility limit in the short term and then decreased over longer time scales, with larger decreases for higher total aluminium concentrations. Higher concentrations of precipitated $Al(OH)_3$ facilitate more rapid adsorption of $Al(OH)_4^-$ and or/ dissolved $Al(OH)_3$ (Angel et al., 2014). The variation over 72 h in concentrations of total aluminium observed at the highest concentration tested (10000 µg/L) is most likely an artefact of sampling, as swirling of the flasks could result in the majority of the precipitate ending up in the vortex region, whilst sedimentation may occur in the short time between swirling and sampling, thus variations in sampling could result in different samples of particulates per volume.

The changes in the forms of aluminium with time and concentration demonstrates the importance of quantifying the total and dissolved concentrations at the beginning and end, and if possible during the bioassay. Several authors have recommended that toxicity be expressed as the measured initial metal concentration rather than the nominal metal concentration to account for losses by adsorption to the container surface (Batley et al., 1999; Stauber and Davies, 2000; Warne et al., 2014). Due to the dynamic nature of aluminium speciation in marine waters this study also expressed toxicity as the measured time averaged metal concentration to account for the fluctuation of the different forms of aluminium over the duration of the bioassays.

4.2 Toxicity of aluminium to marine diatoms

The most striking observation to emerge from comparison of the concentration-response curves with both total and dissolved (<0.45 µm) aluminium was evidence for different chemical forms of aluminium eliciting toxicity in each of these three diatom species. For *C. closterium*, toxicity appeared to be more strongly related to dissolved aluminium: the onset of toxicity, as defined by decreasing growth rate, occurred below the onset of precipitation (500 µg/L). For *M. polymorphus*, it is most

likely that both dissolved and precipitated aluminium contributed to toxicity: the onset of toxicity occurred just prior to the onset of precipitation (500 µg/L). For *P. tricornutum*, toxicity appears to be more strongly related to precipitated aluminium: the onset of toxicity occurred above the onset of precipitation (500 µg/L) and showed the best relationship with total aluminium. In accordance with the current results Golding et al. (2014) also demonstrated that marine microalgae are sensitive to different forms of aluminium. Their study found that toxicity in the diatom *C. closterium* was due to dissolved aluminium forms, and both dissolved and precipitated aluminium contributed to toxicity in the diatom *M. polymorphus* and the green flagellate alga *Dunaliella tertiolecta*, whereas toxicity to the green flagellate alga *Tetraselmis* sp. was due to precipitated aluminium only.

IC10 and IC50 concentrations based on dissolved (<0.45 µm) aluminium concentration were lower (by a maximum of 8 times) than those based on total aluminium concentrations for all species, with greater deviation between the endpoints calculated with dissolved (<0.45 µm) and total aluminium for the more tolerant species investigated. Only the IC10 for *C. closterium* represents effect due to dissolved aluminum as the response of all other species to aluminium exposure occurred just prior to or above the onset of precipitation and are therefore a response to both dissolved and precipitated aluminium (of varying proportions). Thus toxicity endpoints based on total aluminium in the current study are more robust. IC10 and IC50 values based on measured time-averaged aluminium concentrations for the most sensitive species *C. closterium* were slightly lower (by 1.3 times) than those based on initial measured aluminum concentrations whereas for the more tolerant species, *M. polymorphus* and *P. tricornutum* values were in good agreement (Table 3.2). Since the toxicity endpoints based on measured time-averaged aluminum concentrations account for the loss of aluminium due to adsorption to flask glass walls and the fluctuations of the different forms of aluminium over the duration of the bioassays these values were also considered to be more robust.

Reported IC10 concentrations for the effects of aluminium on growth inhibition over 72 h based on time-averaged concentrations of measured total aluminium, span three orders of magnitude for *C. closterium, M. polymorphus* and *P. tricornutum* (80, 540, 2100 µg/L, respectively). All test conditions were identical and thus illustrate that, even in a single class, there are large variations in sensitivity of microalgae to aluminium. This finding suggests that while *C. closterium* is particularly sensitive to aluminium toxicity, diatoms as a taxonomic group are not.

In the current study no pattern in toxicity was observed according to cell shape or cell size with both the most sensitive and the most tolerant species being the larger pennate diatoms. In a similar study, that investigated the effect of biotic factors on the sensitivity of copper to marine microalgae, Levy et al. (2007) found that interspecies differences in copper sensitivity were not related to cell size, cell

wall type or taxonomic group. In contrast, this study found that diatoms with a siliceous cell wall (*C. closterium* and *M. polymorphus*) were more sensitive to aluminium than the diatom with a weakly siliceous cell wall (*P. tricornutum*). It is important to consider that we are studying the effect on only three different species. To be able to establish a trend a larger sample size would need to be investigated. This result should be further investigated as a possible mechanism of toxicity has been suggested involving aluminum uptake or adsorption by diatoms may delay frustule dissolution rates and/or solubility in marine diatoms disrupting cell division (Gensemer and Playle, 1999).

There is little published information available on the toxicity of aluminum to diatoms in marine waters to compare with the data in the present study. Harford et al. (2011) determined 72-h IC10 value of 14 µg/L aluminium using the tropical strain of *C. closterium*. The 72-h IC10 concurred with that derived in the study by Golding et al. (2014) of 18 µg/L using the temperate strain of the same species. These values however, are much lower than that found in this study of 80 µg/L aluminium. Both these aforementioned studies were performed at an initial cell density of 10^4 cells/mL while the current study began with more environmentally realistic densities of 10^3 cells/mL.

4.2.1 Effect of initial cell density

The results obtained in the cell density experiments with *C. closterium* and *M. polymorphus* showed that aluminium toxicity increased when initial cell density was increased. Contrary to the results of the current study, previous research on the influence of initial cell density on metal toxicity to microalgae has reported reduced toxicity with increased initial cell density (Vasseur et al., 1988; Stratton and Giles, 1990; Moreno-Garrido et al., 2000; Franklin et al., 2002; Debelius et al., 2009). Vasseur et al. (1988) found that for bioassays with *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*), when the initial cell density was increased from 10^4 to 10^6 cell/mL the concentration of metal required to inhibit growth by 50% increased from 10 to 280 µg/L, 90 to 365 µg/L and 46 to 300 µg/L for copper, zinc and cadmium, respectively. Vasseur et al. (1988) suggested that decreased toxicity with increasing initial cell density could be due to less metal bound per cell, resulting in lower toxicity. Likewise, Franklin et al. (2002), found that for bioassays with the freshwater chlorophytes, *Chlorella* sp. and *S. capricornutum*, copper toxicity decreased when the initial cell density increased from 10^2 to 10^5 cells/mL (copper concentrations required to inhibit growth by 50% increased from 4.6 to 16 µg/L and from 6.6 to 17 µg/L for each species respectively). Franklin et al. (2002) measured the concentration of intra- and extra-cellular copper at the different cell densities and found that copper toxicity decreased at higher initial cell densities due primarily to greater copper adsorption by algal cells resulting in depletion of the equilibrium concentration of dissolved copper in solution.

It is possible that at higher cell densities, exudate concentrations are higher, causing a measurable increase in the complexation of aluminium. The difference between <0.025 µm and <0.45 µm dissolved aluminium was assumed to be aluminium complexed by algal exudates. For *C. closterium* and *M. polymorphus*, complexed aluminium was similar regardless of the initial cell density. For *P. tricornutum* however, an increase in complexed aluminium was found at the highest initial cell density of 10^5 cells/mL. *P. tricornutum* was the only diatom that showed greater tolerance to aluminium at the initial cell density of 10^5 cells/mL. These results could suggest that for *P. tricornutum* algal exudates may play a role in complexing aluminium and reducing aluminium-cell binding at higher initial cell densities leading to the decreased toxicity observed for this species. However, it is important to note this experiment was only performed once and should be repeated. More in-depth research on this relationship needs to be undertaken before the association between algal exudate production and aluminium availability are more clearly understood.

4.2.2 Environmental relevance

Aluminium was toxic to *C. closterium*, with effects on growth as low as 80 µg/L. Aluminium has shown to reach concentrations of 83 µg/L in the industrialized harbors and coastal waters of Port Curtis, QLD, Australia (Angel et al., 2012). In contrast Aluminium had no inhibitory effect on algal growth of *M. polymorphus* or *P. tricornutum* at environmentally relevant concentrations as aluminium precipitates are not likely to be present in the marine environment unless catastrophically high levels of aluminium are released into seawater, e.g. in the case of a spill. The current low reliability environmental concern level (0.5 µg/L) and the recommended revised high reliability water quality guideline (24 µg total Al/L for 95% species protection) for aluminium in marine waters would afford protection to all three marine diatom species.

4.3 Mechanisms of aluminium toxicity

This study successfully optimised SYTOX Green staining conditions (concentration and time) for the three marine diatoms which were then used to investigate membrane permeability. The optimized staining conditions for the three marine diatoms present in this study are similar to those of other diatom species described in the literature. Chang et al. (2011) used an incubation time of 7 min with a concentration of 0.1 µM SYTOX Green with the freshwater diatom *Nitzschia palea.* Timmermans et al., (2007) optimised the staining conditions for three species of marine diatoms (*Chaetoceros brevis, Chaetoceros calcitran* and *Thalassiosira antarctica*) and found an incubation time of 10 min with 0.5 µM SYTOX Green was best.

No effects of plasma membrane permeability were observed for any of the diatom species suggesting mechanisms of aluminium toxicity to diatoms do not involve compromising the plasma membrane. The current study provides evidence for different marine diatoms responding to different forms of aluminium. Use of these three species in additional studies would enhance our understanding of why some species are sensitive to dissolved aluminum and others are tolerant. Further studies focused on quantifying and analyzing the uptake, and possible bioaccumulation of aluminum are required to determine whether the tolerant species are not able to readily take up aluminium or if there are intracellular detoxification mechanisms protecting these species.

At present, we do not know which molecular forms of aluminium are capable of crossing membranes (Taylor et al., 2000). Essential metals, such as copper, zinc and iron are required for normal cellular functioning in microalgal cells, and thus there are specific mechanisms in place to regulate their cellular uptake and accumulation (Bridges and Zalups, 2005). In contrast nonessential metals such as aluminum, mercury and cadmium have no know biological function and accordingly no specific, dedicated mechanisms have evolved for their uptake into cells (Bridges and Zalups, 2005).

While it is generally acknowledged that most metals enter algal cells by cation transport systems, in a few cases, trace metal internalization has been shown to occur via anionic transporters (Campbell et al., 2002). In such cases, metals are piggy backed across transporters meant for low molecular weight ligands and the uptake of inorganic nutrients (e.g. phosphate, sulfate) and organic anions (e.g. carboxylate anion, amino acids) (Campbell et al., 2002). Fortin and Campbell (2001) found that silver-thiosulfate complexes are transported across the plasma membrane via sulfate/thiosulfate transport systems. Errécalde and Campbell (2000) found that enhanced bioavailability of cadmium in the presence of citrate could be explained by transport of charged cadmium-citrate complex through anionic channels meant for citrate. Jensen et al. (2003) found that uptake of manganese, presumably in the form of manganese-phosphate complex, was accomplished via a cell surface anionic transporter meant for phosphate. Aluminate could similarly be transported into the cell via anion membrane channels.

The overall hydrophobic nature of the plasma membrane, results in only neutral or non-polar molecules being capable of crossing into the cytosol by passive diffusion (based on the concentration gradient between external and internal compartments and a partition coefficient between the aqueous and lipid phase) (Worms et al., 2006). For metals, this mode of transport had been shown to be relevant for dithiocarbamate metal complexes (e.g. pesticides), organomercury and neutral silver chloride complexes (Worms et al., 2006). The neutral but polar aluminium hydroxide complex is not likely to passively diffuse across the cell membrane as non-polar complexes do but there may be

compatible channels or proteins that permit transport across the cell membrane that are, as yet, unknown.

Indirect mechanisms of aluminium toxicity in marine waters may involve competition for binding sites with essential anionic nutrients such as phosphate, nitrate and silicate in the surrounding media. However, aluminate out-competing nutrient uptake is unlikely given that the algal bioassays were enriched with nitrate and phosphate to ensure nutrients were not limiting. It would be useful to repeat the growth-rate inhibition bioassays at low phosphate concentrations to investigate whether aluminium toxicity is influence by phosphate concentration as this has been shown to affect the toxicity of other metals such as arsenic. Levy et al. (2005) found that an increase in phosphate concentration (0.15-1.5 mg PO_4^{3-}/L) decreased the arsenic toxicity in *Monoraphidium arcuatum*. This study also demonstrated that at the higher phosphate concentration intra- and extra-cellular arsenate decreased indicating competition between arsenate and phosphate for cellular uptake.

4.4 Limitations

All experiments of the current study were performed under controlled laboratory conditions. At present it is not known how aluminium toxicity to marine microalgae is affected by abiotic factors such as, temperature, light, water quality characteristics, or exposure duration which fluctuate in the natural marine environment. The response of *C. closterium* does not appear to be affected by temperature. Harford et al. (2011) determined the IC10 of the tropical strain (32°) of *C. closterium* to be 14 (3-25) µg/L (95% confidence limit) which concurs with that found in the study of Golding et al. (2014).of 18 (11-26) µg/L for the temperate (21°C) strain. No other studies investigating the role of abiotic factors on the toxicity of aluminium to marine microalgae could be found in the literature.

Furthermore, at present our understanding of how biotic factors such as exposure route, physiological status and interactions with other organisms effect aluminium toxicity is limited. Results of our investigations are from single-species tests, whereas in the natural marine environment many species co-exist. It is known that different microorganisms compete with one another for available nutrients and attempt to dominate by producing exudates that may be toxic to other species. Work by Vasconcelos and Leal (2008) found that significant changes of nickel, cadmium, iron, zinc and manganese uptake by *P. tricorumutum* were observed in the presence of exudates of different algal species and both the intensity of production and composition of exudates released by *P. tricornutum* were markedly influenced by the presence of other exudates of other algae. Thus while negligible exudates that complexed dissolved aluminium were found to be produced by *C. closterium*, *M. polymorphus* or *P. tricornutum* at the initial cell density of 10^3 cells/mL in our single species test it is

unclear whether the presence of other species may prompt the production of exudates with metal binding capacity.

In natural environments individual contaminants rarely occur alone. In the natural environment potential antagonistic, synergistic, additive effects or ameliorative interactions between metals may occur. Saçan et al. (2007) found that individual exposure of aluminum and lead both caused alterations in the ultrastructure of the marine alga *Dunaliella tertiolecta* but in combination aluminium and lead appeared to act synergistically on the cell membrane leading to cell membrane lysis. Moreover, in the current study bioassays, test algae were exposed instantaneously to high aluminium concentrations which places maximum stress on the cells' adaptive mechanisms (Stauber and Davies, 2000). Although this may represent initial discharges at the point source of anthropogenic contamination, more gradual increases in aluminium concentrations in coastal marine environment receiving anthropogenic discharges are expected to occur over time. Whether natural populations may slowly adapt to increased aluminium concentrations is not known.

Further laboratory investigations with the three marine diatoms of this study would help address some of these limitations and provide important information on the environmental relevance of laboratory bioassays for use in extrapolation of results to natural systems.

5 CONCLUSION

This study set out to relate aluminium sensitivity of three species of marine diatoms (*Ceratoneis closterium, Minutocellus polymorphus,* and *Phaeodactylum tricornutum*) to aluminium speciation and investigate the mechanisms of toxicity of aluminium to the diatoms. One of the most significant findings to emerge from this study was that different forms of aluminium were responsible for eliciting toxicity in each of the three species of marine diatoms investigated. Toxicity in the most sensitive diatom, *C. closterium*, was shown to be due to the dissolved aluminium forms of aluminate ($Al(OH)_4^-$) and aluminium hydroxide $Al(OH_3^0)$, while both dissolved and precipitated aluminium forms contributed to toxicity in *M. polymorphus*. In contrast, aluminium toxicity to *P. tricornutum* was due to precipitated aluminium.

There was a varied response in growth rate sensitivity to aluminium between diatom species with the most sensitive species being *C. closterium* (72-h exposure inducing 10% inhibition of growth rate (72-h IC10) = 80 (55-100) µg Al/L (95% confidence limits)) followed by *M. polymorphus* (72-h IC10 = 540 (460-600) µg Al/L) and *P. tricornutum* (72-h IC10 = 2100 (2000-2200) µg Al/L) based on time averaged concentrations of measured total aluminium. The current finding suggests that while *C. closterium* is particularly sensitive to aluminium toxicity, diatoms as a taxonomic group are not.

The project was designed to also investigate where biotic factors such as the ultrastructure of the cell wall, cell shape or cell size were related to interspecies sensitivity to aluminium. The evidence from this study suggests that interspecies sensitivity to aluminium in marine diatoms, as measured by growth-rate inhibition, were not related to cell size or cell shape. A relationship between the ultrastructure of the cell wall and aluminium toxicity was observed however, with the two diatoms species with a siliceous cell wall more sensitive than the diatom with the weakly siliceous cell wall. Further research needs to examine more closely the links between cell ultrastructure composition and aluminium toxicity.

Furthermore, sensitivity of *C. closterium* and *M. polymorphus* was also found to be influenced by initial cell density, with aluminium toxicity increasing with increasing initial cell density from 10^3 to 10^5 cells/mL. This result is contrary to that observed for other metals investigated in the literature, namely copper, cadmium and zinc and should be investigated further to provide additional insight into the mechanisms of aluminium toxicity.

This study successfully optimised SYTOX Green staining conditions (concentration and time) for the three marine diatoms which were then used to investigate membrane permeability. No effects on

plasma membrane permeability were observed for any of the species suggesting mechanisms of aluminium toxicity to diatoms does not involve compromising the plasma membrane. The precise mechanism of aluminium toxicity in marine diatoms remains to be elucidated, but the results of this study suggest that there are specific mechanisms of toxicity that include both dissolved and precipitated aluminium forms. Further research is needed to understand how the different forms of aluminium interact with the surface of algal cells and whether aluminium is transported into algal cells or exerts it toxicity extracellular. Further investigations using the three marine diatoms of this study would allow for comparisons to be draw between a sensitive species, a moderately tolerant species and a tolerant species and identify possible resistance mechanisms.

Lastly, consideration and understanding of aluminium speciation and the factors affecting aluminium bioavailability in the marine environment are essential to develop bioassays that yield meaningful data. Appropriate interpretation of toxicity data requires an understanding of the specific limitations of the investigation. The present study is only applicable for aluminium toxicity at pH 8.2 and the ionic composition of seawater with enriched phosphate and nitrate.

Overall this study has used an integrated approach, combining chemical analysis, 72-h growth-rate inhibition bioassays and the use of the fluorochrome SYTOX Green to increase the current understanding of aluminium toxicity to marine diatoms. The results of this study provide an additional robust account of the sensitivity of marine diatoms to aluminium exposure and add to the emerging body of literature on the effects of aluminium on marine organisms.

REFERENCES

Adams, M. S. and Stauber, J. L. (2004). Development of a whole-sediment toxicity test using a benthic marine microalga. *Environmental Toxicology and Chemistry*, **23**(8): 1957-1968.

Angel, B. M., Apte, S. C., Batley, G. E. and Golding, L. A. (2014). The role of solubility in the speciation of aluminium in marine waters. *Environmental Toxicology and Chemistry*,(submitted).

Angel, B. M., Jarolimek, C. V., King, J. J., Hales, L. T., Simpson, S. L., Jung, R. F. and Apte, S. C. (2012). Metal concentrations in the waters and sediments of Port Curtis, Queensland, CSIRO Wealth from Oceans Flagship Report, Lucas Heights, NSW, Australia.

ANZECC/ARMCANZ (2000). Australian and New Zealand Guidelines for Fresh and Marine Water Quality, Australian and New Zealand Environment and Conservation Council/Agricultural and Resource Management Council of Australia and New Zealand, Canberra, Australia.

Apte, S. C., Batley, G. E., Szymczak, R., Rendell, P. S., Lee, R. and Waite, T. D. (1998). Baseline trace metal concentrations in New South Wales coastal waters. *Marine and Freshwater Research*, **49**(3): 203-214.

Batley, G. E., Apte, S. C. and Stauber, J. L. (1999). Applicability of aquatic toxicity data for the derivation of water quality guidelines for metals. *Marine and Freshwater Research*, **50**: 729-738.

Bridges, C. C. and Zalups, R. K. (2005). Molecular and ionic mimicry and the transport of toxic metals. *Toxicology and Applied Pharmacology*, **204**(3): 274-308.

Brown, M. T., Lippiatt, S. M. and Bruland, K. W. (2010). Dissolved aluminum, particulate aluminum, and silicic acid in northern Gulf of Alaska coastal waters: Glacial/riverine inputs and extreme reactivity. *Marine Chemistry*, **122**(1–4): 160-175.

Bruland, K. W. and Lohan, M. C. (2003). *6.02 - Control of trace metals in seawater*. In Elderfield, H., The Oceans and Marine Geochemistry, Treatise on Geochemistry. Elsevier, **6:** 23-47.

Campbell, P. G. C., Errécalde, O., Fortin, C., Hiriart-Baer, V. P. and Vigneault, B. (2002). Metal bioavailability to phytoplankton—applicability of the biotic ligand model. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **133**(1–2): 189-206.

Caschetto, S. and Wollast, R. (1979). Vertical distribution of dissolved aluminium in the mediterranean sea. *Marine Chemistry*, **7**(2): 141-155.

Debelius, B., Forja, J. M., DelValls, Á. and Lubián, L. M. (2009). Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicology and Environmental Safety*, **72**(5): 1503-1513.

Dixit, S. S., Smol, J. P., Kingston, J. C. and Charles, D. F. (1992). Diatoms - Powerful indicators of environmental-change. *Environmental Science & Technology*, **26**(1): 22-33.

Elkins, K. M. and Nelson, D. J. (2002). Spectroscopic approaches to the study of the interaction of aluminum with humic substances. *Coordination Chemistry Reviews*, **228**(2): 205-225.

Errécalde, O. and Campbell, P. G. C. (2000). Cadmium and zinc bioavailability to *Selenastrum capricornutum* (Chlorophyceae): accidental metal uptake and toxicity in the presence of citrate. *Journal of Phycology*, **36**(3): 473-483.

Fortin, C. and Campbell, P. G. C. (2001). Thiosulfate Enhances Silver Uptake by a Green Alga: Role of Anion Transporters in Metal Uptake. *Environmental Science & Technology*, **35**(11): 2214-2218.

Franklin, N. M., Stauber, J. L., Apte, S. C. and Lim, R. P. (2002). Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. *Environmental Toxicology and Chemistry*, **21**(4): 742-751.

Franklin, N. M., Stauber, J. L. and Lim, R. P. (2001). Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. *Environmental Toxicology and Chemistry*, **20**(1): 160-170.

Franklin, N. M., Stauber, J. L. and Lim, R. P. (2004). Development of multispecies algal bioassays using flow cytometry. *Environmental Toxicology and Chemistry*, **23**(6): 1452-1462.

Gehlen, M., Beck, L., Calas, G., Flank, A. M., Van Bennekom, A. J. and Van Beusekom, J. E. E. (2002). Unraveling the atomic structure of biogenic silica: evidence of the structural association of Al and Si in diatom frustules. *Geochimica et Cosmochimica Acta*, **66**(9): 1601-1609.

Gensemer, R. W. and Playle, R. C. (1999). The Bioavailability and Toxicity of Aluminum in Aquatic Environments. *Critical Reviews in Environmental Science and Technology*, **29**(4): 315-450.

Golding, L. A., Angel, B. M., Batley, G. E., Apte, S. C., Krassoi, R. and Doyle, C. J. (2014). Derivation of a water quality guideline for aluminium in marine waters. *Environmental Toxicology and Chemistry*,(accepted).

Harford, A. J., Hogan, A. C., Tsang, J. J., Parry, D. L., Negri, A. P., Adams, M. S., Stauber, J. L. and van Dam, R. A. (2011). Effects of alumina refinery wastewater and signature metal

constituents at the upper thermal tolerance of: 1. The tropical diatom Nitzschia closterium. *Marine Pollution Bulletin,* **62**(3): 466-473.

Hydes, D. J. (1979). Aluminum in seawater: Control by inorganic processes. *Science (New York, N.Y.),* **205**(4412): 1260-1262.

Hydes, D. J. (1983). Distribution of aluminium in waters of the North East Atlantic 25°N to 35°N. *Geochimica et Cosmochimica Acta,* **47**(5): 967-973.

Hydes, D. J., de Lange, G. J. and de Baar, H. J. W. (1988). Dissolved aluminium in the Mediterranean. *Geochimica et Cosmochimica Acta,* **52**(8): 2107-2114.

Hydes, D. J. and Liss, P. S. (1977). The behaviour of dissolved aluminium in estuarine and coastal waters. *Estuarine and Coastal Marine Science,* **5**(6): 755-769.

Jensen, L. T., Ajua-Alemanji, M. and Culotta, V. C. (2003). The *Saccharomyces cerevisiae* High Affinity Phosphate Transporter Encoded by PHO84 Also Functions in Manganese Homeostasis. *Journal of Biological Chemistry,* **278**(43): 42036-42040.

King, J. J. (2013). Ultratrace determination of aluminium and gallium in marine waters. Honours Thesis, University of Wollongong, Wollongong, Australia.

Kramer, J., Laan, P., Sarthou, G., Timmermans, K. R. and de Baar, H. J. W. (2004). Distribution of dissolved aluminium in the high atmospheric input region of the subtropical waters of the North Atlantic Ocean. *Marine Chemistry,* **88**(3–4): 85-101.

Levy, J. L., Angel, B. M., Stauber, J. L., Poon, W. L., Simpson, S. L., Cheng, S. H. and Jolley, D. F. (2008). Uptake and internalisation of copper by three marine microalgae: Comparison of copper-sensitive and copper-tolerant species. *Aquatic Toxicology,* **89**(2): 82-93.

Levy, J. L., Stauber, J. L., Adams, M. S., Maher, W. A., Kirby, J. K. and Jolley, D. F. (2005). Toxicity, biotransformation, and mode of action of arsenic in two freshwater microalgae (*Chlorella* Sp. and *Monoraphidium arcuatum*). *Environmental Toxicology and Chemistry,* **24**(10): 2630-2639.

Levy, J. L., Stauber, J. L. and Jolley, D. F. (2007). Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity. *Science of The Total Environment,* **387**(1–3): 141-154.

Mackin, J. E. and Aller, R. C. (1984). Processes affecting the behavior of dissolved aluminum in estuarine waters. *Marine Chemistry,* **14**(3): 213-232.

Maring, H. B. and Duce, R. A. (1987). The impact of atmospheric aerosols on trace metal chemistry in open ocean surface seawater, 1. Aluminum. *Earth and Planetary Science Letters,* **84**(4): 381-392.

Martin, R. B. (1991). Fe^{3+} and Al^{3+} hydrolysis equilibria. Cooperativity in Al^{3+} hydrolysis reactions. *Journal of Inorganic Biochemistry,* **44**: 141-147.

Measures, C. I., Brown, M. T. and Vink, S. (2005). Dust deposition to the surface waters of the western and central North Pacific inferred from surface water dissolved aluminum concentrations. *Geochemistry, Geophysics, Geosystems,* **6**(9): Q09M03.

Middag, R., van Slooten, C., de Baar, H. J. W. and Laan, P. (2011). Dissolved aluminium in the Southern Ocean. *Deep Sea Research Part II: Topical Studies in Oceanography,* **58**(25–26): 2647-2660.

Millero, F. J., Woosley, R., Ditrolio, B. and Waters, J. (2009). Effect of Ocean Acidification on the Speciation of Metals in Seawater. *Oceanography,* **22**(4): 72-85.

Monteiro, C. M., Castro, P. M. L. and Malcata, F. X. (2012). Metal uptake by microalgae: underlying mechanisms and practical applications. *Biotechnology progress,* **25**(2): 299-311.

Moore, R. M. (1981). Oceanographic distributions of zinc, cadimum, copper and aluminium in waters of the central Arctic Ocean. *Geochimica et Cosmochimica Acta,* **45**: 2475-2482.

Moran, S. B. and Moore, R. M. (1988a). Evidence from mesocosm studies for biological removal of dissolved aluminium from sea water. *Nature,* **335**(6192): 706-708.

Moran, S. B. and Moore, R. M. (1988b). Temporal variations in dissolved and particulate aluminum during a spring bloom. *Estuarine, Coastal and Shelf Science,* **27**(2): 205-215.

Moran, S. B., Moore, R. M. and Westerlund, S. (1992). Dissolved aluminum in the Weddell Sea. *Deep Sea Research Part A. Oceanographic Research Papers,* **39**(3–4): 537-547.

Moreno-Garrido, I., Lubián, L. M. and Soares, A. M. V. M. (2000). Influence of Cellular Density on Determination of EC50 in Microalgal Growth Inhibition Tests. *Ecotoxicology and Environmental Safety,* **47**(2): 112-116.

Namieśnik, J. and Rabajczyk, A. (2010). The Speciation of Aluminum in Environmental Samples. *Critical Reviews in Analytical Chemistry,* **40**(2): 68-88.

Nordmyr, L., Åström, M. and Peltola, P. (2008). Metal pollution of estuarine sediments caused by leaching of acid sulphate soils. *Estuarine, Coastal and Shelf Science,* **76**(1): 141-152.

Orians, K. J. and Bruland, K. W. (1986). The biogeochemistry of aluminum in the Pacific Ocean. *Earth and Planetary Science Letters,* **78**(4): 397-410.

Pilson, M. E. Q. (2013). *An introduction to the chemistry of the sea*. Cambridge, UK, Cambridge University Press.

Rodgher, S., Espindola, E. L. G., Simoes, F. C. F. and Tonietto, A. E. (2012). Cadmium and Chromium Toxicity to *Pseudokirchneriella subcapitata* and *Microcystis aeruginosa*. *Brazilian Archives of Biology and Technology,* **55**(1): 161-169.

Saçan, M. T., Oztay, F. and Bolkent, S. (2007). Exposure of *Dunaliella tertiolecta* to Lead and Aluminum: Toxicity and Effects on Ultrastructure. *Biological Trace Element Research,* **120**(1-3): 264-272.

Sato, M., Murata, Y., Mizusawa, M., Iwahashi, H. and Oka, S. (2004). A simple and rapid dual-fluorescence viability assay for microalgae. *Microbial Culture Collection,* **20**(2): 53-59.

Stauber, J. L. (1995). Toxicity testing using marine and freshwater unicellular algae. *Australasian Journal of Ecotoxicology*, **1**: 15-24.

Stauber, J. L. and Davies, C. M. (2000). Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. *Environmental Reviews,* **8**(4): 255-301.

Stauber, J. L., Franklin, N. M. and Adams, M. S. (2002). Applications of flow cytometry to ecotoxicity testing using microalgae. *Trends in Biotechnology,* **20**(4): 141-143.

Stauber, J. L., Franklin, N. M. and Adams, M. S. (2005). *Mircoalgal toxicity testing using flow cytometry*. In Blaise, C. and Ferard, J. F., Small-scale Freshwater Toxicity Investigations. Springer, Dordrecht, Netherlands, **1:** 203-241.

Stoffyn, M. (1979). Biological control of dissolved aluminum in seawater: Experimental evidence. *Science (New York, N.Y.),* **203**(4381): 651-653.

Stoffyn, M. and Mackenzie, F. T. (1982). Fate of dissolved aluminum in the oceans. *Marine Chemistry,* **11**(2): 105-127.

Stratton, G. and Giles, J. (1990). Importance of bioassay volume in toxicity tests using algae and aquatic invertebrates. *Bulletin of Environmental Contamination and Toxicology,* **44**(3): 420-427.

Tashyreva, D., Elster, J. and Billi, D. (2013). A Novel Staining Protocol for Multiparameter Assessment of Cell Heterogeneity in Phormidium Populations (Cyanobacteria) Employing Fluorescent Dyes. *Plos One,* **8**(2).

Taylor, G. J., McDonald-Stephens, J. L., Hunter, D. B., Bertsch, P. M. and et al. (2000). Direct measurement of aluminum uptake and distribution in single cells of Chara corallina. *Plant Physiology,* **123**(3): 987-996.

vanLoon, G. W. and Duffy, S. J. (2011). *Environmental chemistry, a global perspective*. New York, Oxford University Press.

Vasconcelos, M. T. S. D. and Leal, M. F. C. (2008). Exudates of different marine algae promote growth and mediate trace metal binding in *Phaeodactylum tricornutum*. *Marine Environmental Research,* **66**(5): 499-507.

Vasseur, P., Pandard, P. and Burnel, D. (1988). Influence of some experimental factors on metal toxicity to *Selenastrum capricornutum*. *Toxicity Assessment,* **3**(3): 331-343.

Warne, M. S. J., Batley, G. E., Braga, O., Chapman, J. C., Fox, D. R., Hickey, C. W., Stauber, J. L. and Van Dam, R. (2014). Revisions to the derivation of the Australian and New Zealand guidelines for toxicants in fresh and marine waters. *Environmental Science and Pollution Research*, **21**(1): 51-60.

Wilson, R. W. (2011). *2 - Aluminum*. In Wood, C. M., Farrell, A. P. and Brauner, C. J., Fish Physiology. Academic Press, **31B:** 67-123.

Wilson, S. P. and Hyne, R. V. (1997). Toxicity of Acid-Sulfate Soil Leachate and Aluminum to Embryos of the Sydney Rock Oyster. *Ecotoxicology and Environmental Safety*, **37**(1): 30-36.

Worms, I., Simon, D. F., Hassler, C. S. and Wilkinson, K. J. (2006). Bioavailability of trace metals to aquatic microorganisms: importance of chemical, biological and physical processes on biouptake. *Biochimie*, **88**(11): 1721-1731.

APPENDIX A QUALITY ASSURANCE AND CONTROL

A.1 Sensitivity of diatom cultures to reference toxicant

Copper was used as a reference toxicant to assess the performance and response of the diatom cultures over time to ensure that the algae were responding to a known reference toxicant in a reproducible way. As part of each bioassay, three flasks were prepared at a single copper concentration that would bring about ≥ 50% reduction in growth rate compared to the control, based on 72-h toxicity endpoints presented in Levy et al. (2007). The response of the three marine diatoms to the copper reference toxicant are shown in Table A.1.1. The results across all bioassays were comparable with each species exhibiting similar sensitivity in repeat reference toxicity tests throughout the project.

Table A.1.1 Response of marine diatoms to copper reference toxicant to assess the health and sensitivity of the cultures over time

Test date	Copper exposure concentration[a] (µg/L)	Growth rate (% of the control)
Ceratoneis closterium		
15/07/2014	15.2	14 ± 11
29/07/2014	13.1	18 ± 0.8
12/08/2014	13.6	16 ± 0.5
Minutocellus polymorphus		
29/07/2014	1.4	7 ± 3
05/08/2014	1.6	19 ± 0.02
Phaeodactylum tricornutum		
22/07/2014	14.0	2 ± 0.02
05/08/2014	13.7	9 ± 0.02
12/08/2014	13.9	8 ± 0.03

[a] Measured dissolved (<0.45 µm) copper concentrations

A.2 Validation of changes to standard method

For all three diatom species initial bioassays were performed at two treatment volumes (50 mL and 100 mL) concurrently to validate that no change in control growth rate or sensitivity to aluminium resulted from the change to the standard protocol. Control growth rates and IC10/IC50 values for each marine diatom are presented in Table A.2.1. Control growth rates were acceptable (Stauber et al., 2005) with values ≥ 1 doublings per day and the difference between the two volume treatments < 0.3 doublings per day, which is within the natural variation observed for replicate tests. No difference was observed between effect concentrations (IC10 and IC50) based on total aluminium as evidenced by the overlap between the 95% CL of the two treatments. Overall the results across the two treatments concurred indicating that volume had no effect on the growth or sensitivity of the three diatoms to aluminium.

Table A.2.1 Marine diatom control growth rates, percent coefficient and sensitivity to aluminium in two treatment volumes (50 and 100 mL) over 72-h growth-rate inhibition bioassays

Treatment volume	Control performance		Measured total aluminium[a] (µg/L)	
	Growth rate[b]	%CV[c]	IC10[d] (95% CL)	IC50[d] (95% CL)
Ceratoneis closterium				
50 mL	2.39 ± 0.07	3	103 (39–187)	4409 (2795–5917)
100 mL	2.11 ± 0.05	2	129 (0–217)	2209 (0–6478)
Minutocellus polymorphus				
50 mL	2.14 ± 0.03	2	734 (593-900)	4323 (3863-4640)
100 mL	2.20 ± 0.03	1	736 (644-892)	4358 (3782-4922)
Phaeodactylum tricornutum				
50 mL	1.47 ± 0.06	4	1850 (1071-2003)	6341 (5892-6582)
100 mL	1.46 ± 0.05	4	1788 (1117-2036)	6081 (5397-6841)

[a] Toxicity endpoints calculated from measured total aluminium concentrations

[b] Control growth rate in doublings/day

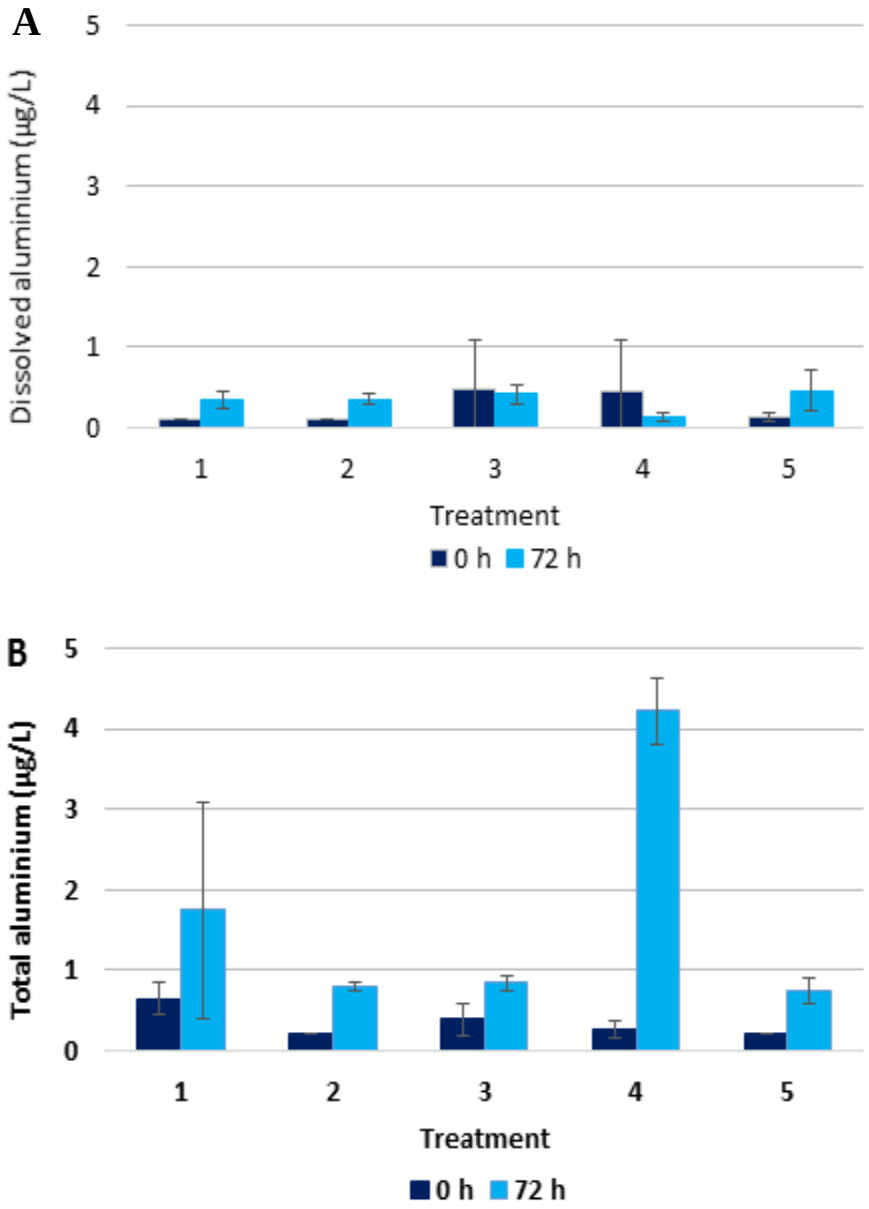
[c] %CV: percent coefficient of variation of control growth rate

[d] IC10/IC50: Concentration of aluminium to cause a 10% and 50% inhibition of the diatom cell growth rate over 72-h respectively

A.3 Aluminium contamination

An experiment was set up to monitor changes in dissolved and total aluminium concentration over 72 h to investigate possible sources of ambient particulate aluminium contamination. Control seawater was utilised for all treatments (aluminium-free filtered seawater plus nitrate and phosphate). Five treatments, each containing three replicate flasks were set up in the same area where bioassays were conducted: 1) flasks with lids that were not removed, 2) flasks without lids, 3) flasks with lids that were removed daily in the lab for 30 min, 4) flasks with *C. closterium* and lids removed daily in the lab for 30 min, and 5) flasks with lids that were removed daily in a laminar flow cabinet (i.e. a dust-free environment) for 30 min. Replicates were subsampled at 0 h and 72 h for total and dissolved (<0.45 µm) aluminium and preserved with 5% and 2% HNO_3 (v/v) (Tracepur, Merck), respectively.

The concentration of dissolved aluminium was similar in all treatments, and no difference was measured between 0 and 72 h. The following treatments - flasks without lids (treatment 2), flasks with lids that were removed daily in the lab for 30 min (treatment 3) and flasks with lids that were removed daily in a laminar flow cabinet (treatment 5) - all had measured total aluminium concentrations less than the treatment where the lids were not removed at all (treatment 1) over the duration of the experiment indicating that any potential ambient particulate aluminium contamination was negligible (Figure A.3.1). The flasks with the most increase in aluminium were the flasks with *C. closterium* and lids removed daily in the lab for 30 min (treatment 4). As previously mentioned, removing the lids daily in the lab in the absence of algae (treatment 3) did not lead to increased aluminium concentration being observed relative to lids not being removed (treatment 1). It is possible that the microalgae themselves may have a role in the increased aluminium concentrations observed, such as by facilitating the dissolution of Al from the borosilicate glassware (borosilicate glass contains 2% Al_2O_3) and adsorbing any release aluminium so it is in the particulate form.



Treatment:

1) Flasks with lids that were not removed
2) Flasks without lids
3) Flasks with lids that were removed daily in the lab for 30 min
4) Flasks with *Ceratoneis closterium* and lids removed daily in the lab for 30 min
5) Flasks with lids that were removed daily in a laminar flow cabinet (i.e. dust free environment)

Figure A.3.1 Concentration of aluminium in control flasks as a function of time: A) dissolved (<0.45 µm) aluminium and B) total aluminium. Each point represents the mean of 3 replicates ± the standard deviation (*vertical error bars*). Where no error bars are shown, they are within the points

A.4 Adsorption of aluminium to glass walls of test vessel

Removal of Al (via adsorption to glass flask walls) from the bioassay test solutions in the absence of algae was examined over 72 h. Four aluminium treatments (10-10000 µg Al/L) and a control (aluminium-free filtered seawater plus nitrate and phosphate) were prepared. Triplicate 100 mL samples of the test solutions were dispensed into test flasks for each treatment and control. This was

repeated for a volume of 50 mL. Immediately after an aliquot was dispensed into the test flask, the replicate was sub-sampled for total aluminium and preserved with 5% (v/v) HNO_3 (Tracepur, Merck). This represents total aluminium at time 0 h. Sub-sampling for total aluminium was repeated at 2 h and 72 h. After the 72-h sub-sample was collected, the remaining solution in the flask was acidified to 5% with (v/v) HNO_3 (Tracepur, Merck) to recover adsorbed aluminium from the flask walls, left for 24 h swirling occasionally and sub-sampled again at 96 h.

The majority of aluminium loss occurred within the first 2 h (Table A.4.1) with nominal concentrations ≤ 100 µg/L being most affected. Further loss of aluminium occurred between 2 h and 72 h for all treatments except the control, with greater losses for treatments ≤ 100 µg/L. At low concentrations (≤ 100 µg/L nominal) aluminium at 96 h was greater than the initial aluminium concentration introduced to the flask (Table A.4.1) indicating a source of aluminium contamination on acidification – most likely from aluminium dissociation from the Coatasil silanising solution or from the borosilicate glass itself. Thus recovery of aluminium by acidification was unable to confirm aluminium was lost to the flask wall at lower concentrations.

Table A.4.1 Decrease in total aluminium concentrations after 2 h and 72 h in the absence of test organisms

Initial (0 h) aluminium concentrations (µg/L)		Loss of aluminium[b] (%)		Aluminium concentration at 96 h (µg/L)
Nominal	Measured[a]	2 h	72 h	
100 mL treatment				
Control	1.2 ± 0.2	95	55	32 ± 2
10	10.0 ± 0.7	48	87	40 ± 10
100	89 ± 3	28	46	109 ± 1
1000	893 ± 7	3	9	905 ± 7
10000	9100 ± 100	6	10	8470 ± 50
50 mL treatment				
Control	2 ± 1	74	23	73 ± 3
10	9.9 ± 0.3	67	88	80 ± 4
100	87.7 ± 0.4	41	68	160 ± 10
1000	980 ± 10	5	15	973 ± 7
10000	9200 ± 100	1	14	8500 ± 100

[a] mean ± SD of three replicates

[b] loss of aluminium as a % of the initial measured aluminium concentration

APPENDIX B SUPPLEMENTARY MATERIAL

B.1 Changes in total aluminium over 72-h bioassays with *M. polymorphus* and *P. tricornutum*

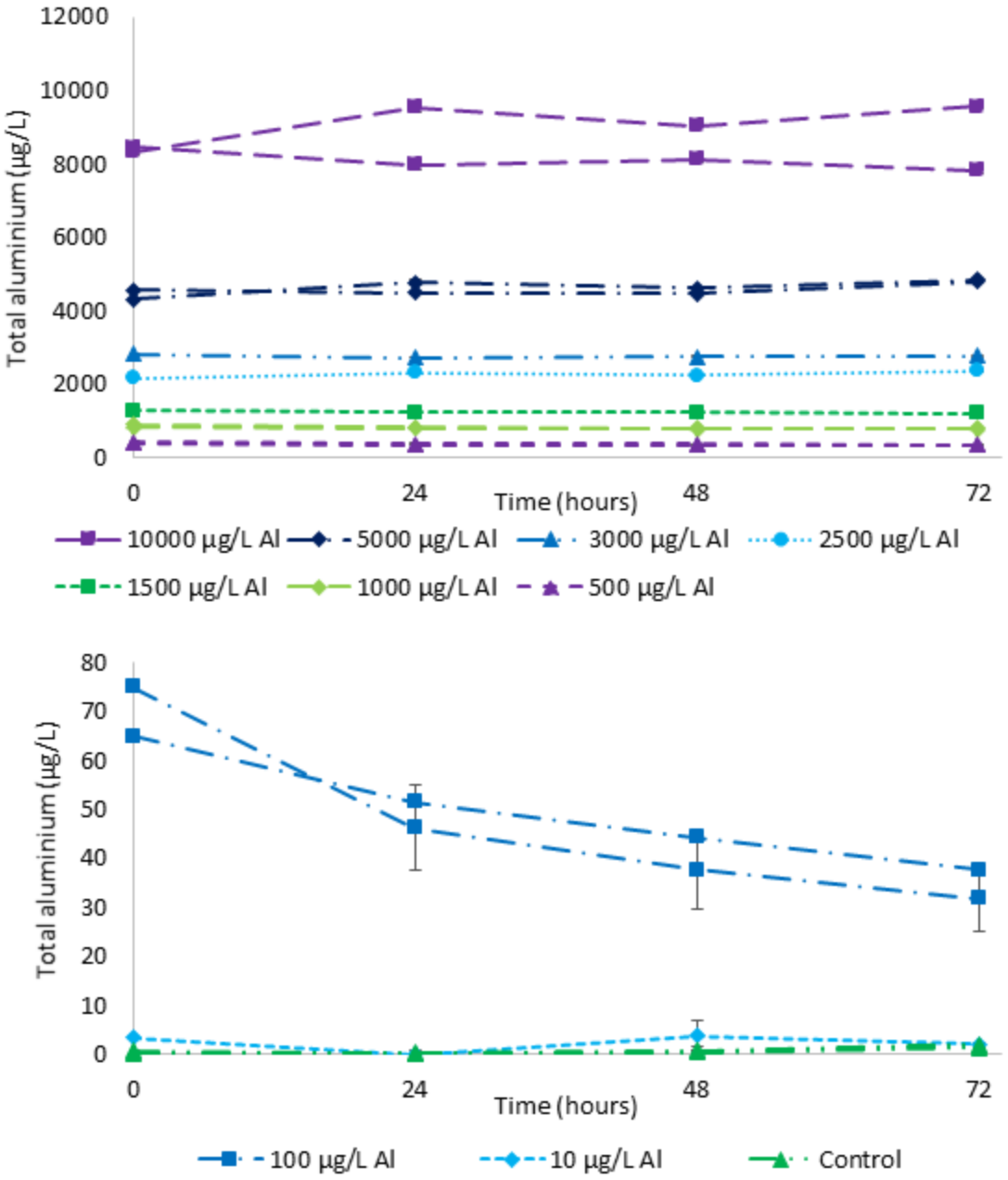


Figure B.1.1 The concentration of total aluminium in bioassay test solutions with *Minutocellus polymorphus* as a function of time for nominal aluminium concentrations: A) 500 to 10000 µg/L and B) 0 to 100 µg/L (mean ± standard deviation, n=3, vertical error bars. Concentrations of 100, 500, 1000, 5000, and 10000 µg/L were tested in more than 1 bioassay

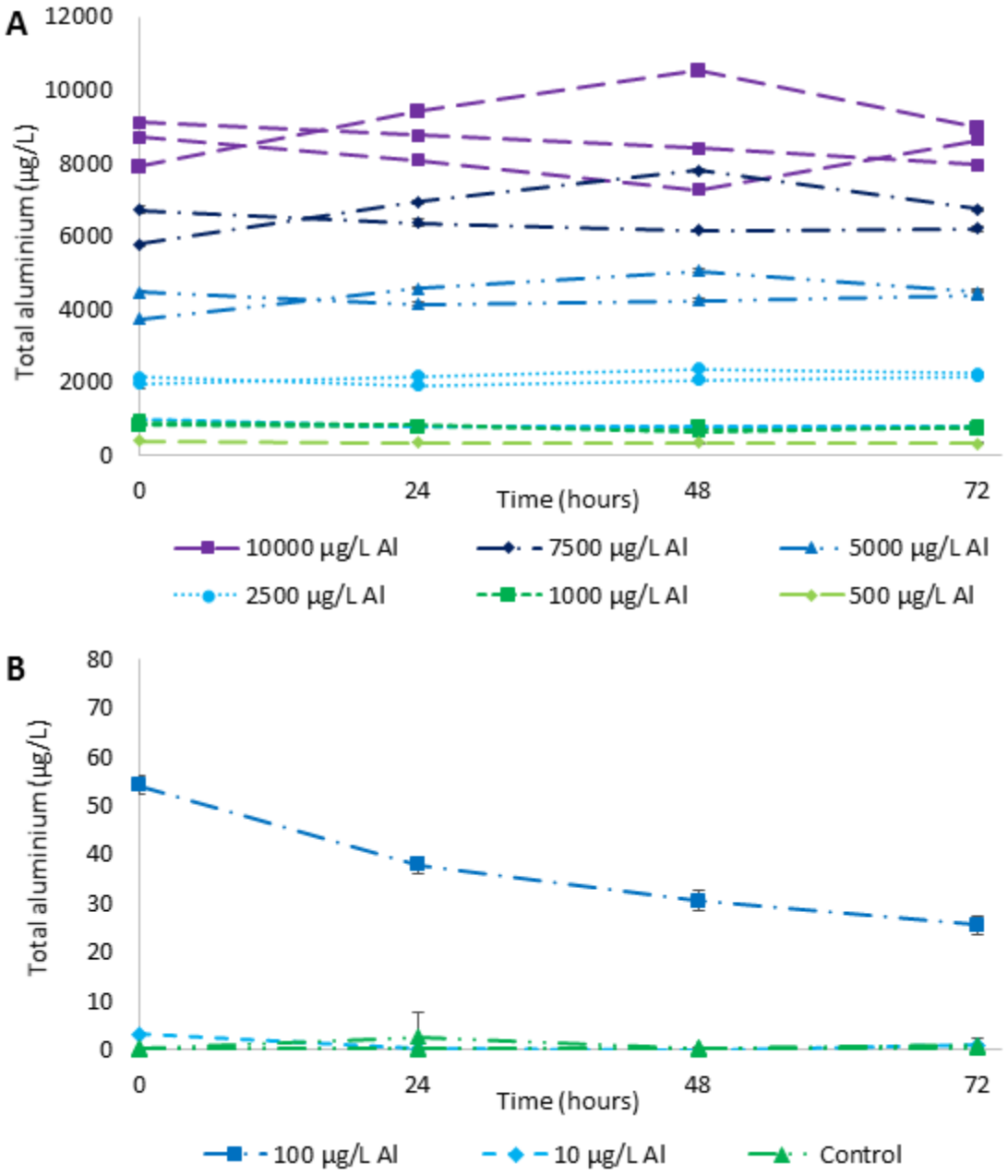


Figure B.1.2 The concentration of total aluminium in bioassay test solutions with *Phaeodactylum tricornutum* a function of time for nominal aluminium concentrations: A) 500 to 10000 µg/L and B) 0 to 100 µg/L (mean ± standard deviation, n=3, vertical error bars). Concentrations of 1000, 2500, 5000, 7500, and 10000 µg/L were tested in more than 1 bioassay

B.2 Changes in dissolved aluminium over 72 h bioassays with *M. polymorphus* and *P. tricornutum*

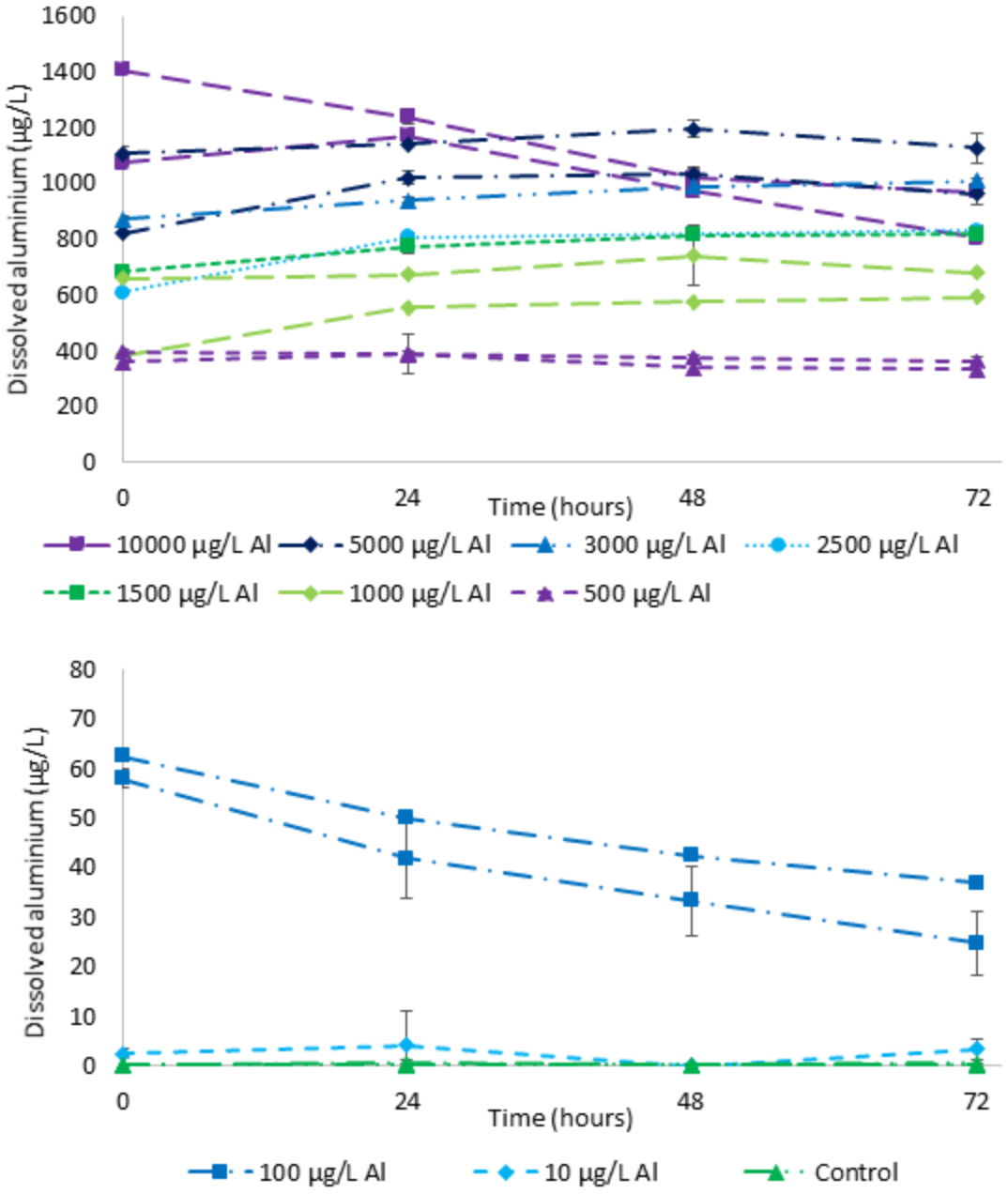


Figure B.2.1 The concentration of dissolved aluminium (<0.45 µm) in bioassay test solutions with *Minutocellus polymorphus* as a function of time for nominal aluminium concentrations:. A) 500 to 10000 µg/L and B) 0 to 100 µg/L (mean ± standard deviation, n=3, vertical error bars). Concentrations of 100, 500, 1000, 5000 and 10000 µg/L were tested in more than 1 bioassay

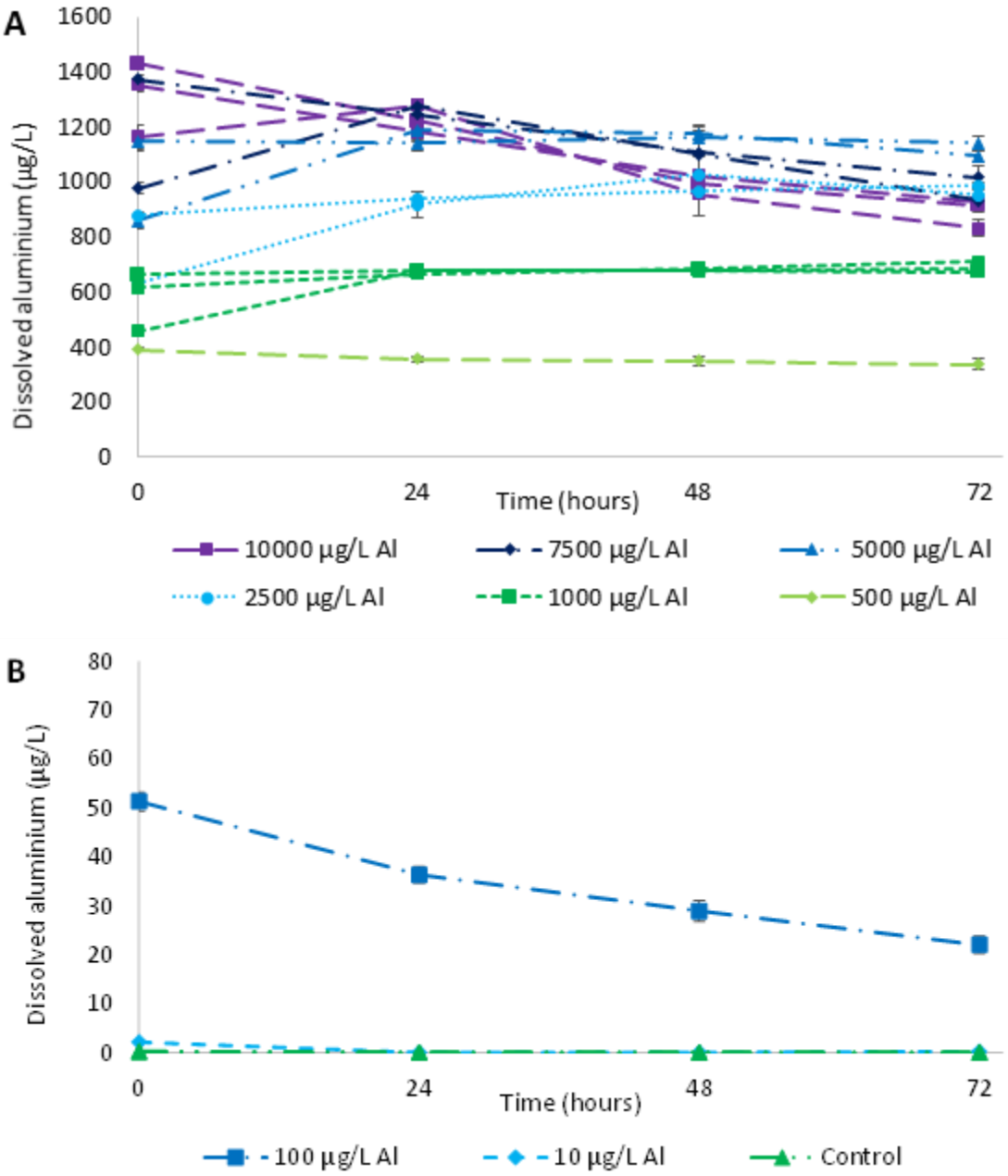


Figure B.2.2 The concentration of dissolved aluminium (<0.45 µm) in bioassay test solutions with *Phaeodactylum tricornutum* as a function of time for nominal aluminium concentrations: A) 500 to 10000 µg/L and B) 0 to 100 µg/L (mean ± standard deviation, n=3, vertical error bars). Concentrations of 1000, 2000, 5000, 7500 and 10000 µg/L were tested in more than 1 bioassay

B.3 Concentration response curves of marine diatoms as a function of total and dissolved (<0.45 µm) aluminium concentrations at 0 h, 72 h and time-averaged

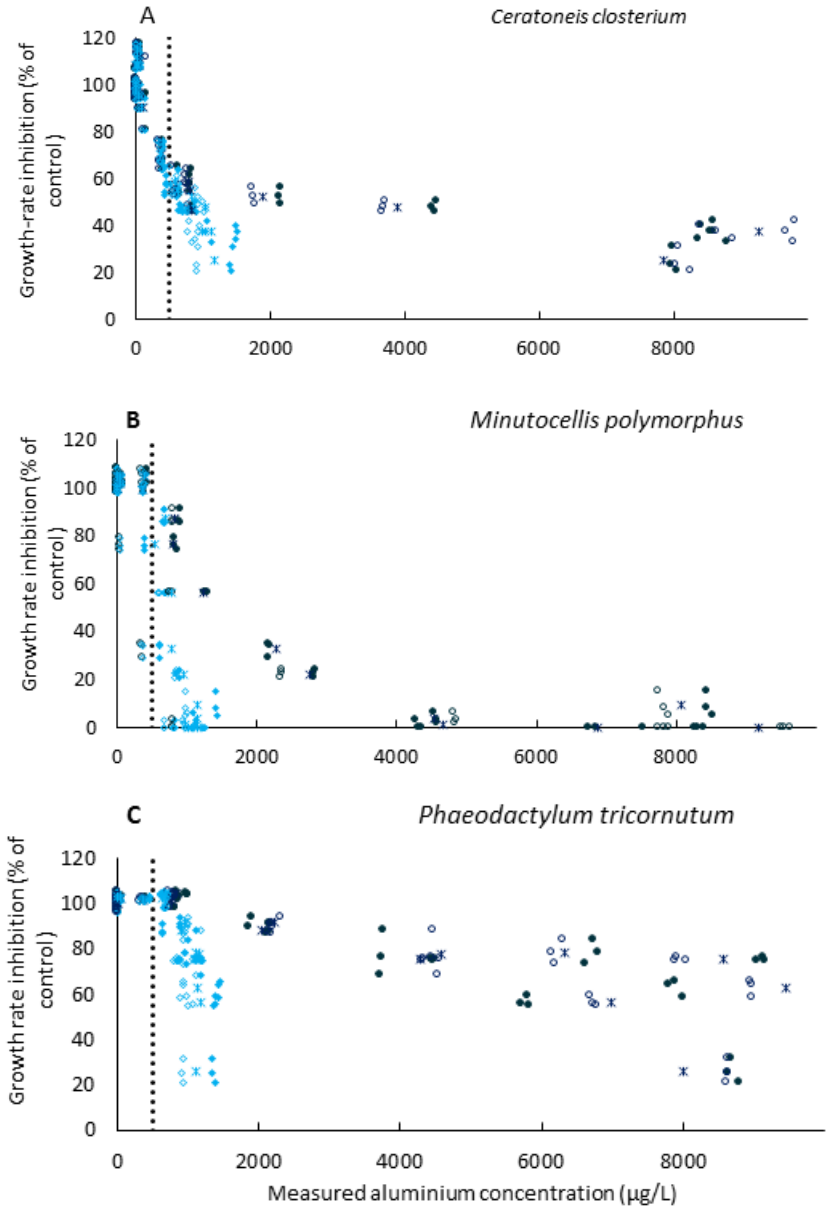


Figure B.3.1 The effect of aluminium on the growth rate of three marine diatoms after 72-h exposure (A) *Ceratoneis closterium*, (B) *Minutocellus polymorphus* and (C) *Phaeodactylum tricornutum*. Growth rate for each replicate was plotted as a percentage of the mean control growth rate. The results for each of the diatom species are the combination of ≥ 3 separate bioassays pooled together where each point represents 1 replicate. The black dotted line (x=500 µg Al/L) represents the approximate aluminium concentration above which precipitation begins. Total (*dark blue*) and dissolved (<0.45 µm) (*light blue*) aluminium at the beginning of the bioassay (0 h , *closed symbol*), end of the bioassay (72 h, *open symbol*) and time-averaged (mean of 0, 24, 48 and 72 h concentrations, asterisk)

B.4 Representative flow cytometric dot plots revealing clear separation of auto green fluorescence and enhanced SYTOX Green fluorescence due to increased membrane permeability under optimised staining conditions

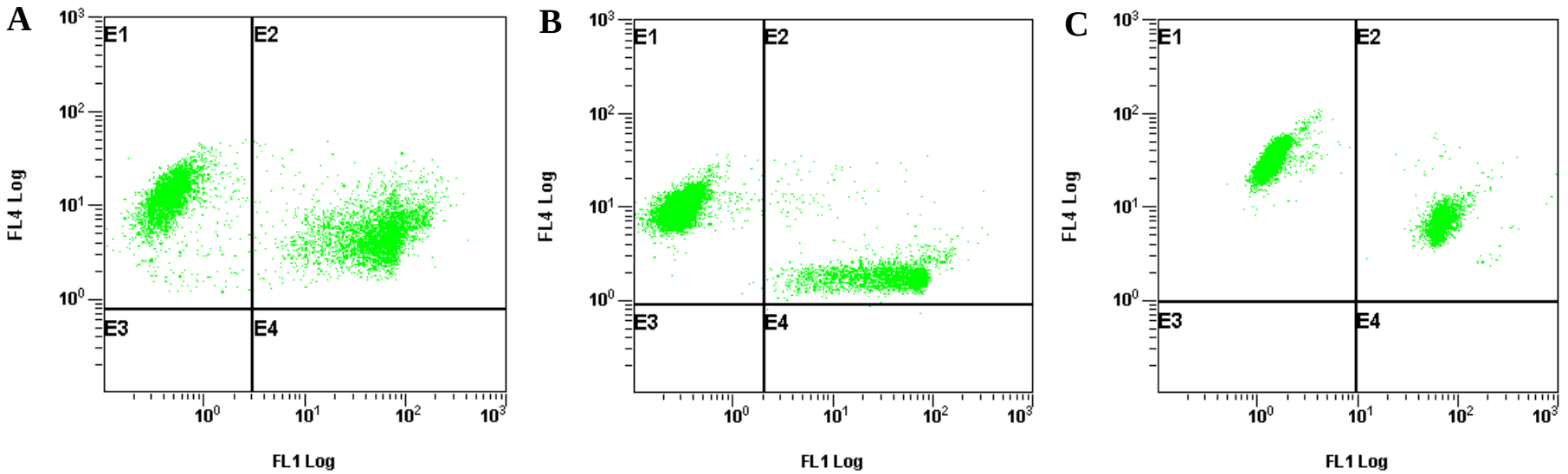


Figure B.4.1 Examples of flow cytometric dot-plots showing auto green fluorescence of healthy cells (E1) and increased SYTOX Green fluorescence (E2) due to membrane permeability of heat-treated cells under optimized staining conditions (0.5 µM SYTOX Green and 5 min incubation in the dark) for: A) *Ceratoneis closterium*, B) *Minutocellus polymorphus* and C) *Phaeodactylum tricornutum*

B.5 Representative flow cytometric dot-plots revealing enhanced SYTOX Green fluorescence of positive control cells, auto green fluorescence of control cells and increased spread of auto green fluorescence of aluminium treated cells

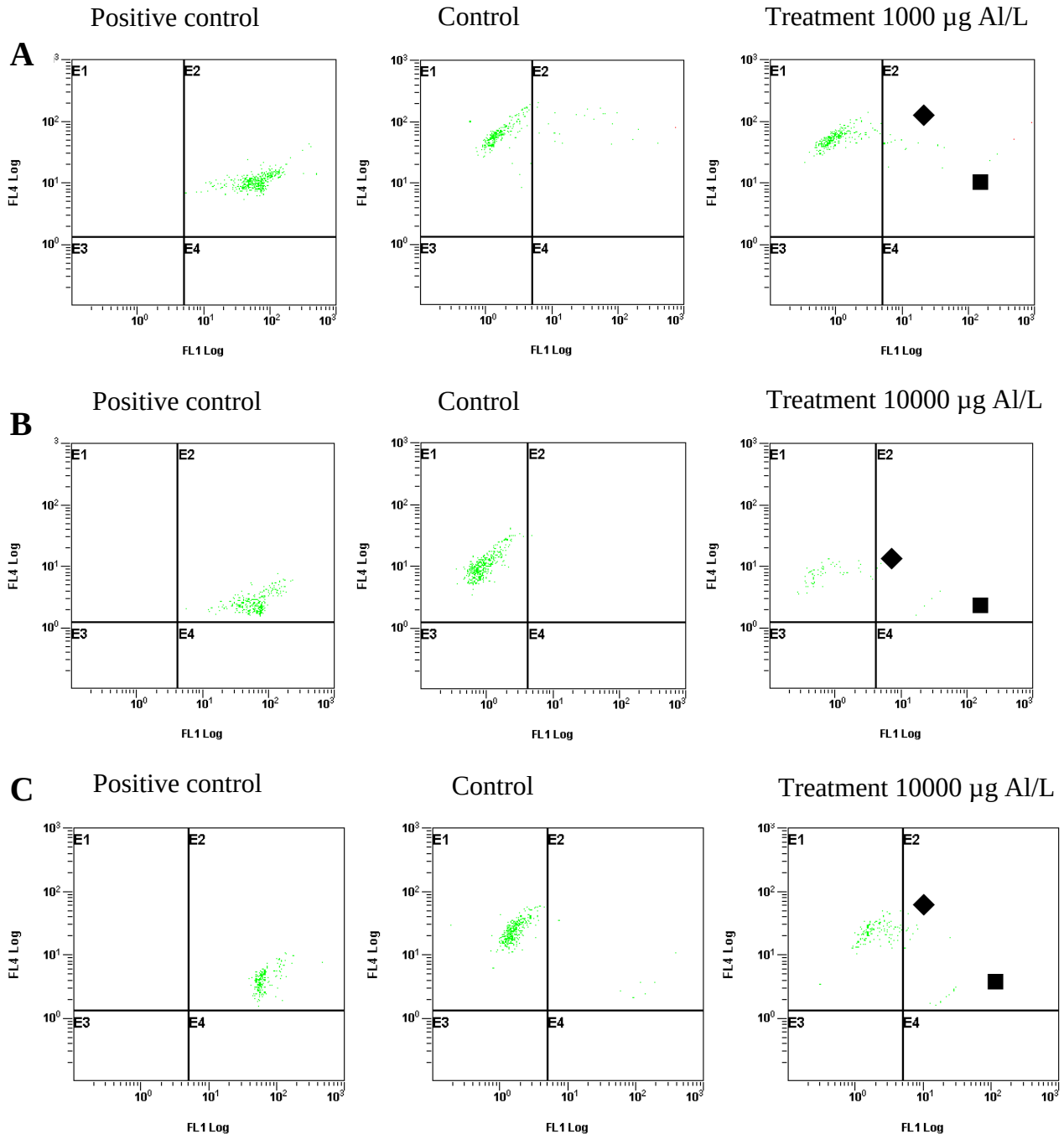


Figure B.5.1 Examples of flow cytometric dot-plots for A) *Ceratoneis closterium*, B) *Minutocellus polymorphus* and C) *Phaeodactylum tricornutum*. E1 region captures auto green fluorescence of healthy cells while E2 region captures SYTOX Green fluorescence indicative of a permeabilized plasma membrane. Symbols represent increased spread of auto green fluorescence into E2 region (◆) and the location where enhanced SYTOX Green fluorescence would be expected to show up, indicating increased membrane permeability (■)

B.6 Diatom membrane permeability at 48 and 72 h

Table B.6.1 Membrane permeability of marine diatoms exposed to aluminium for 48 h measured by SYTOX Green binding

Nominal Al treatment (µg/L)	Membrane permeability (% of cells in E2)		
	Ceratoneis closterium	*Minutocellus polymorphus*	*Phaeodactylum. tricornutum*
Positive control	99.7 ± 0.4	99.3 ± 0.3	99.9 ± 0.1
Control	1.5 ± 0.6	0.17 ± 0.06	0.5 ± 0.2
100	0.9 ± 0.5	0.07 ± 0.01	0.45 ± 0.08
500	1.7 ± 0.8	0.11 ± 0.02	0.31 ± 0.08
1000	6.47 ± 1.7	3.7 ± 0.4	1.1 ± 0.4
2500	14 ± 2	3.8 ± 0.5	3.1 ± 0.5
5000	12.2 ± 0.8	9 ± 3	3.4 ± 0.8
10000	6 ± 2	4 ± 4	3 ± 2

[a] Mean ± SD, n ≥ 3

Table B.6.2 Membrane permeability of marine diatoms exposed to aluminium for 72 h measured by SYTOX Green binding

Nominal Al treatment (µg/L)	Membrane permeability (% of cells in E2)		
	Ceratoneis closterium	*Minutocellus polymorphus*	*Phaeodactylum. tricornutum*
Positive control	99.6 ± 0.3	98.5 ± 0.5	99.8 ± 0.1
Control	1.1 ± 0.4	0.3 ± 0.1	0.4 ± 0.1
100	1.0 ± 0.6	0.3 ± 0.1	0.5 ± 0.2
500	1.8 ± 0.3	0.2 ± 0.1	0.43 ± 0.04
1000	6 ± 4	3.5 ± 0.3	1.0 ± 0.3
2500	30 ± 1	4.9 ± 0.8	3.1 ± 0.8
5000	24 ± 3	11 ± 4	4 ± 2
10000	6 ± 1	4 ± 2	3.0 ± 0.8

[a] Mean ± SD, n ≥ 3